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Asociación Española de
Enfermos de Glucogenosis

GLUCOGENOSIS

CONGRESO INTERNACIONAL 2013

Editores:

Alberto Molares Vila
Jesús Sueiro Justel
Javier Fernández Salido
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Alberto Molares Vila
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(Editores)

Índice

I. Últimos Avances

1. CIBERER Biobank: una plataforma de apoyo a la investigación en enfermedades raras 11
   Salvador Martí, Virgina Corrochano, José María Millán y Francesc Palau.

2. Unidad de Atención a Pacientes Adultos con errores congénitos del metabolismo 23
   Francesc Cardellach, Josep Mª Grau, Carmen Visiedo, María Forga, Antònia Ribes

3. Bortezomib in the rapid reduction of high sustained antibody titers in disorders treated with therapeutic protein: lessons learned from pompe disease 27
   Suhrad G. Banugaria, Sean N. Prater, Judeth K. McGann, Jonathan D. Feldman, Jesse A. Tannenbaum, Carrie Bailey, Renuka Gera, Robert L. Conway, David Viskochil, Joyce A. Kobori, Amy S. Rosenberg, Priya S. Kishnani

4. Allosteric Regulation of Glycogen Synthase Controls Glycogen Synthesis in Muscle 39
   Michale Bouskila, Roger W. Hunter, Adel F.M. Ibrahim, Lucie Delattre, Mark Peggie, Janna A. van Diepen, Peter J. Voshol, Jørgen Jensen, Kei Sakamoto

5. Characterization of a canine model of glycogen storage disease type IIIa 53
   Haiqing Yi, Beth L. Thurberg, Sarah Curtis, Stephanie Austin, John Fyfe, Dwight D. Koeber, Priya S. Kishnani, Baodong Sun

6. Molecular Mechanism by Which AMP-Activated Protein Kinase Activation Promotes Glycogen Accumulation in Muscle 63
   Roger W. Hunter, Jonas T. Treebak, Jørgen F.P. Wojtaszewski, Kei Sakamoto

7. Successful immune tolerance induction to enzyme replacement therapy in CRIM-negative infantile Pompe disease 75
   Yoav H. Messinger, Nancy J. Mendelsohn, William Rhead, David Dimmock, Eli Hershkovitz, Michael Champion, Simon A. Jones, Rebecca Olson, Amy White, Cara Wells, Deeksha Bali, Laura E. Case, Sarah P. Young, Amy S. Rosenberg, Priya S. Kishnani

II. Guías actualizadas

8. Guía informativa para la glucogenosis Tipo IX (Deficiencia de Fosforilasa Kinasa) 6ª edición. 89

Laura Castells Molines, Leonor Fernández Marcos, Alberto Molares Vila

9. Guía nutricional en glucogenosis 103

Miguel Carnero Gregorio, Luisa Fernanda Pérez Méndez

I - Últimos avances

CIBERER BIOBANK: UNA PLATAFORMA DE APOYO A LA INVESTIGACIÓN EN ENFERMEDADES RARAS

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La investigación sobre enfermedades raras representa un nuevo concepto social y sanitario que intenta centrar e integrar su atención en los aspectos médicos, científicos y sociales de los pacientes y de las familias que sufren trastornos de baja prevalencia y que históricamente no han sido bien reconocidos por la sociedad y los sistemas de salud.
El Centro de Investigación Biomédica en Red de Enfermedades Raras o CIBERER es un consorcio dependiente del Instituto de Salud Carlos III (ISCIII) - Ministerio de Economía y Competitividad que nace en 2006 con la voluntad de coordinar y promover la actividad de investigación cooperativa en el ámbito de enfermedades raras (ER). Para ello, cuenta con 60 grupos de investigación clínicos y básicos, pertenecientes a 29 instituciones referentes de investigación españolas repartidos en 8 Comunidades Autónomas. Más de 700 científicos e investigadores conforman su equipo humano entre personal adscrito y contratado. Las actividades de investigación del CIBERER están organizadas en siete Programas Temáticos Médicos y constituyen el motor de coordinación y actividad científica del CIBERER.
Esta iniciativa constituye en gran medida la respuesta dada por el Gobierno a la problemática de las ER, reflejada en 2005 en la Ponencia al Senado, que durante un año analizó la situación de los pacientes con ER, constituida en el seno de la Comisión conjunta de la Comisión de Sanidad y Consumo y de la Comisión de Trabajo y Asuntos Sociales.
El CIBERER es un centro orientado hacia el desarrollo y la implementación de la investigación cooperativa en el ámbito de las ER, favoreciendo la investigación biomédica básica, clínica y epidemiológica, poniendo un énfasis especial en trasladar la investigación desde el laboratorio a la cabecera del paciente y responder científicamente a las preguntas nacidas de la interacción médico-enfermo.
El CIBERER es un Centro de Investigación en Red con una estructura innovadora. Se constituye sobre la base de la trayectoria y el potencial investigador de los grupos de investigación que lo conforman. El consorcio proporciona coordinación estratégica, recursos humanos y materiales, además de un entorno colaborativo donde explotar las sinergias propias del gran potencial de conocimiento multidisciplinar y complementario. Los grupos se integran en programas científicos donde se coordinan y potencian estas colaboraciones.
Sin embargo, la labor del CIBERER no acaba con la generación del conocimiento. Necesita completarse con la transferencia de éste desde el laboratorio a la clínica y viceversa, respondiendo al concepto global de investigación traslativa o traslacional. Las ER son muchas veces complejas y la aplicación en beneficio del paciente de los resultados de la investigación necesita de la complicidad de los departamentos clínicos ubicados en centros hospitalarios y otros centros sanitarios con los grupos de investigación básica. El CIBERER aglutina estos dos potenciales al incluir grupos de investigación de diferentes ámbitos.

Desde el punto de vista científico, el CIBERER está organizado en 7 Programas Temáticos Médicos. Estos programas tienen como objetivo organizar los grupos sobre la base de las grandes temáticas médicas en las que realizan sus investigaciones. Conceptualmente se consideran siete programas teniendo en cuenta el aspecto fundamental biológico e histórico que caracteriza a cada una de las ER, bien aisladamente, bien como grupo nosológico de enfermedades. Los Programas de Investigación son:

- **Programa de Medicina Genética**, que incorpora enfermedades mendelianas o complejas en las que se afecta un órgano o sistema del cuerpo humano, empleando como criterio fundamental para su incorporación en el programa el factor hereditario.

- Programa de Medicina Metabólica Hereditaria, para el estudio de ER cuyo aspecto fundamental es la alteración de la homeostasis causada por mutaciones en genes relacionados con el metabolismo intermediario.

- Programa de Medicina Mitocondrial, en el que se incluyen las enfermedades que tienen como diana fisiopatológica la mitocondria y afectan el balance bioenergético del individuo. La razón de crearlo independiente del programa de medicina metabólica se basa en el hecho de que, como tales, involucran un número importante de grupos con un historial de trabajo en red muy estrecho.

- Programa de Medicina Pediátrica y del Desarrollo, donde se abordan los trastornos condicionados por un desarrollo embrionario anómalo, indistintamente de la causa, que conllevan una malformación congénita o un trastorno del desarrollo cognitivo, de especial relevancia en la infancia y durante la época de crecimiento y desarrollo del individuo.

- Programa de Patología Neurosensorial, que aborda ER que tienen una afectación sobre los órganos sensoriales, tales como hipoacusias congénitas o las distrofias retinianas.

- Programa de Medicina Endocrina, en el que se engloban los trastornos originados por disfunción hormonal relacionadas con la hipófisis y sus órganos diana.

- Programa de Cáncer Hereditario y Síndromes Relacionados, para el estudio de los mecanismos fisiopatológicos y las bases genéticas del cáncer hereditario así como otros síndromes relacionados, en los cuales la inestabilidad genética es un componente fundamental en la aparición o progresión de la enfermedad.

PLATAFORMAS DE APOYO A LA INVESTIGACIÓN

La estructuración de los programas en el CIBERER es compleja, en gran medida debido

a la propia idiosincrasia del campo de las ER, ámbito de la medicina y de la salud pública que abarca más de 6.000 entidades nosológicas con un componente de transversalidad en el conjunto de los sistemas orgánicos humanos.
Para poder resolver de una manera científica, lógica y operativa esta complejidad, el CIBERER ha optado por una aproximación en la que los Programas de Investigación (PdI) se apoyan en instrumentos de investigación biomédica, concretamente los Proyectos Intramurales Biomédicos en ER (PIBER), y las Plataformas Instrumentales Transversales de apoyo a las ER (PITER).
Las PITER responden a la necesidad de dar apoyo específico a la consecución de los objetivos estratégicos y, en este contexto, es donde el CIBERER Biobank (CBK) se integra dentro de la estructura del CIBERER.
Según la Ley de Investigación Biomédica (Ley 14/2007 de 3 de Julio) un biobanco es un establecimiento público o privado, sin ánimo de lucro, que acoge una colección de muestras biológicas concebida con fines diagnósticos o de investigación biomédica y organizada como una unidad técnica con criterios de calidad, orden y destino.
En la práctica, un biobanco es una plataforma de apoyo a la investigación que actúa de nexo de unión entre donantes, clínicos e investigadores con el propósito de asegurar un tratamiento seguro y eficaz de las muestras biológicas y datos asociados.
Su finalidad sería, por tanto, potenciar y facilitar estudios en diferentes áreas de la investigación biomédica, siendo fundamentales para el desarrollo de la medicina personalizada.
En este sentido, podríamos definir al CBK como un biobanco de carácter público y sin ánimo de lucro puesto en marcha por el CIBERER y ubicado en el Centro Superior de Investigación en Salud Pública (CSISP) de Valencia. El CBK fue creado con una clara aspiración de excelencia y diferenciación, con el objetivo de convertirse en un biobanco que centralice la recepción de muestras de ER en España.
Según la definición de la Unión Europea, ER son aquellas patologías que suponen una amenaza para la vida o un peligro de invalidez crónica y que tienen una prevalencia menor de 5 casos por cada 10.000 habitantes, lo que representa entre el 5 y el 7% de la población de los países desarrollados. A pesar de su baja prevalencia, hay más de 6.000 ER, unos 3 millones de afectados en España y alrededor de unos 30 millones de afectados en los 27 Estados miembros de la Unión Europea. Sin embargo, el concepto de ER es más amplio y con él se hace referencia a trastornos minoritarios, crónicos, generalmente graves, sobre los que hay escasas opciones terapéuticas.
En un elevado porcentaje de estas enfermedades la causa primaria es de origen genético, de ahí la gran importancia de una aproximación genética para entender aspectos fundamentales de la medicina y de la práctica clínica para este grupo de enfermedades.
En este contexto, el CBK fue creado con el objetivo de servir a la investigación diagnóstica y terapéutica, facilitando la disponibilidad de material biológico de alta calidad para llevarla a cabo. Asimismo, pretende solventar uno de los principales problemas con los que se encuentra la investigación en este tipo de patologías: la dispersión de las muestras debida a la baja prevalencia de las ER. Este punto es de especial relevancia en un momento en el que la proliferación de diferentes biobancos podría dificultar la concentración de casos necesarios para llevar a cabo una investigación en ER. De ahí la importancia de

crear un biobanco de muestras de ER y de darle la difusión adecuada entre los profesionales implicados en la asistencia sanitaria de estas patologías.

Estructura organizativa del biobanco

De acuerdo a lo estipulado por la Ley de Investigación Biomédica 14/2007, el biobanco cuenta con un titular, un Director científico, un Responsable del fichero y está adscrito a dos comités externos, el Comité Científico y el Comité de Ética. Cuenta además con un Subdirector Científico, un Coordinador y un Responsable de Laboratorio.
Las tareas de los comités externos son asesorar al director científico y evaluar sin carácter vinculante las solicitudes de cesión de muestras y datos asociados a las mismas por parte del biobanco.

- Comité de Ética: Tiene como misión garantizar el cumplimiento de los principios éticos aplicables a los proyectos de investigación biomédica que incorporen muestras de origen humano del CBK, así como del uso que se haga de las mismas. El CBK se adscribe mediante Convenio al Comité de Ética de la Dirección General de Salud Pública (DGSP) y del CSISP de la Generalitat Valenciana.

- Comité Científico: Tiene como objetivo asesorar a los responsables del CBK sobre la dirección y objetivos científicos del mismo así como desarrollar los estándares de funcionamiento. Asimismo aprueba cualquier transferencia de muestras a terceras partes y asesora en la priorización de la cesión de muestras. El CBK está adscrito al Comité Científico de la Red Valenciana de Biobancos.

El biobanco cuenta además con la supervisión del Comité Científico Asesor Externo (CCAE) del CIBERER, órgano de apoyo y asesoramiento científico general al Consejo Rector, formado por científicos de especial relevancia a nivel internacional en el ámbito de ciencias de la salud que se hayan distinguido por su trayectoria profesional o científica afín a los objetivos del Consorcio.

Marco estratégico

La misión del CIBERER, definida tanto en la convocatoria como en las bases reguladoras y según directrices del ISCIII, es apoyar el estudio y la investigación científica y técnica en el campo de las ER, haciendo hincapié en los aspectos de la investigación genética, molecular, bioquímica y celular, y con el objetivo de mejorar la compresión de las causas y de los mecanismos patogénicos de estos trastornos como pieza fundamental para desarrollar e implementar nuevas técnicas diagnósticas y estrategias terapéuticas.
El CBK se encuentra enmarcado dentro del Plan de actuación CIBERER 2010-2013 como instrumento para potenciar todas sus líneas estratégicas.
Como instrumento del CIBERER, el CBK sirve al objetivo principal del CIBERER: “Convertirse en un centro de referencia español y europeo en la investigación científica

de las bases biológicas y patológicas de las ER - genéticas y adquiridas - con un interés específico en realizar una investigación traslacional que permita la transferencia de conocimiento a los centros sanitarios y a la práctica clínica, en beneficio de los pacientes y sus familiares". Como objetivos específicos del CIBERER encontramos:

a) Contribuir a la investigación en el campo de las ER.

b) Favorecer la resolución de los problemas de asistencia sanitaria relacionados con las ER.

c) Potenciar la participación de los grupos de investigación en actividades científico-técnicas de carácter nacional y especialmente de las incluidas en los programas Marco europeos de I+D+i.

d) Promover la transferencia de resultados de investigación a la sociedad y en especial al Sistema Nacional de Salud y al sector productivo farmacéutico y biotecnológico.

e) Apoyar la divulgación y la participación en actividades docentes relacionadas con el campo de actuación del CIBERER.

Líneas estratégicas

Fomentar el desarrollo de una investigación de excelencia en Enfermedades Raras

La investigación biomédica de excelencia en el seno del CIBERER es el fundamento de la competitividad científica y el desarrollo de programas de innovación del mismo en el ámbito de las ER. Actualmente, hay alrededor de 6.000 ER conocidas según Orphanet y este número sigue en aumento. Este hecho provoca la necesidad de que el CIBERER se especialice en aquellas ER más investigadas por los grupos científicos y que sirva de paraguas y modelos para el conjunto de ER y de que su biobanco dé cabida a muestras de ER en las cuales el CIBERER no investiga con el fin de ponerlas al servicio de toda la comunidad científica.

Las líneas estratégicas del biobanco para los próximos años han sido diseñadas teniendo como referencia el marco estratégico CIBERER, la misión y los objetivos principales que vienen definidos en nuestros estatutos. Además del obligatorio marco estratégico, se han tenido en cuenta los antecedentes, las evaluaciones externas, y la dirección futura del biobanco.

El Plan estratégico estará orientado a facilitar la investigación en el campo de las ER mediante la provisión de muestras y servicios y el fomento de nuevas líneas de acción.

Línea 1. Constituirse como un biobanco de referencia a nivel nacional e internacional

El biobanco del CIBERER se propone centralizar la recepción de muestras de alto valor biológico para la investigación en ER en España contribuyendo así a solventar uno de los problemas principales con los que se encuentra la investigación en este tipo de patologías: la dispersión de muestras debida a la baja prevalencia de las ER. Este repositorio de muestras de ER está a disposición de la comunidad científica, tanto nacional como internacional.

Línea 2. Generar un valor añadido para los grupos CIBERER: el CIBERER Biobank como plataforma tecnológica

El CIBERER potencia la puesta en marcha de plataformas para dar apoyo específico a la actividad investigadora y aprovechar los recursos y sinergias existentes entre el CIBERER y las distintas Instituciones Consorciadas. Como plataforma transversal de apoyo a la investigación, el biobanco se brinda a la colaboración con el resto de grupos del CIBERER para el desarrollo de proyectos conjuntos y la prestación de servicios.

Línea 3. Fomentar y apoyar nuevas líneas de investigación en ER

El biobanco busca adelantarse a las necesidades de sus investigadores, poniendo a punto aquellas técnicas que se prevé contarán con una elevada demanda (células iPS, cultivo de mioblastos, etc.).
Se continuará colaborando con los proyectos de investigación en marcha y se fomentará la participación en otros nuevos.

Línea 4. Mejora continua de procesos e implantación del Sistema de Gestión de Calidad

La calidad ha de ser entendida como la adecuación del producto o servicio ofertado a las necesidades presentes y futuras de los usuarios y se dirige hacia una búsqueda constante de la satisfacción del usuario a través de la adecuación y mejora continua del producto/servicio ofertado. Desde el biobanco se ha procedido a la implantación de un Sistema de Gestión de Calidad (SGC), consistente en la definición y aplicación de una documentación básica necesaria para cumplir con los requisitos de la Norma de referencia.

Línea 5. Incrementar la participación del biobanco en actividades de difusión

Un aspecto muy importante a tener en cuenta por su repercusión sobre el grado de actividad del biobanco es la difusión del mismo entre los distintos agentes implicados (facultativos, pacientes, investigadores, responsables de biobancos, industria biofarmacéutica).

Línea 6. Incrementar la participación del biobanco en actividades formativas

Desde el CBK se promoverá la coorganización y participación en actividades formativas que permitan la profesionalización de los diferentes agentes involucrados en la labor de los biobancos.

Actividades y funcionamiento del biobanco

Los principales objetivos y actividades del CBK están relacionados con la obtención, procesamiento y almacenamiento de muestras clínicas de elevada calidad y sus datos asociados. Son:

- Captación y procesamiento de muestras de alta calidad procedentes de pacientes afectados por alguna ER.
- Almacenamiento de las muestras en condiciones óptimas, garantizando la con-

fidencialidad de las mismas y de todos los datos asociados.

- Retorno de información al profesional sanitario o investigador que ha enviado la muestra y labor de difusión entre los miembros del CIBERER y el resto de grupos de investigación nacionales e internacionales.
- Cesión de muestras a grupos de investigación (públicos o privados) tanto nacionales como internacionales que superen favorablemente la evaluación, por parte del Comité Científico y Ético, del proyecto para el cual solicitan muestras biológicas.
- Fomentar y apoyar nuevas líneas de acción en ER: Implementación de nuevas técnicas con fines terapéuticos y desarrollo de proyectos de epidemiología genética.

Además, el CBK proporciona diferentes tipos de servicios a la comunidad científica:

- Extracción de ADN a partir de sangre periférica.
- Obtención y cultivo de fibroblastos a partir de biopsias de piel.
- Obtención y cultivo de mioblastos a partir de biopsias de músculo.
- Inmortalización de líneas celulares (Linfocitos B, a partir de sangre periférica).
- Implementación de nuevos protocolos, como el de generación de células iPS a partir de fibroblastos procedentes de biopsias de piel.
- Fomento y apoyo de nuevas líneas de acción en ER mediante la aplicación de nuevas técnicas con fines terapéuticos y desarrollo de proyectos de epidemiología genética.

En cuanto al funcionamiento del biobanco, el protocolo a seguir para la donación de muestras consiste en informar al CBK de la intención, por parte del profesional sanitario o investigador, de enviar una o varias muestras de pacientes afectados por una ER. El principal problema ético que se plantea en torno a las muestras biológicas de origen humano se refiere al análisis genético. La mayor parte de las ER tienen una causa genética, lo que conlleva implicaciones que afectan no solo al donante sino a sus familiares. Por tanto, el procedimiento de donación ha de cumplir con lo establecido en la Ley 14/2007 en lo que respecta a la "protección y la integridad del ser humano en cualquier investigación biomédica que implique la realización de análisis genéticos, tratamiento de los análisis genéticos de carácter personal y muestras biológicas de origen humano". Como resultado de esto, cualquier extracción de muestras debe ir acompañada de un consentimiento informado firmado por el donante o su representante legal, precedido por una información adecuada facilitada a los mismos. Dicha información se proporcionará por escrito y comprenderá la naturaleza, importancia, implicaciones y riesgos de la investigación así como los derechos de los donantes. Más concretamente la información hará referencia a:

- Finalidades de la investigación.
- Beneficios esperados.
- Identidad del responsable de la investigación.
- Datos genéticos que se hayan obtenido a partir del análisis de las muestras cedidas.
- Mecanismos para garantizar la confidencialidad de la información obtenida, indicando la identidad de las personas que hayan tenido acceso a los datos de ca-

rácter personal del donante.

La base de datos o LIMS (Laboratory Information Management System) recoge exhaustivamente, además de los datos personales del donante y de los especialistas que los atienden, datos sobre los siguientes aspectos:

- Diagnóstico clínico.
- Tipos de muestra.
- Datos sobre el tipo de muestras disponibles para investigación (ADN, ARN, fibroblastos, etc..).
- Información acerca del objetivo de investigación biomédica.

Todos los contenidos de esta base de datos son tratados con absoluta confidencialidad y el acceso a la misma, por un número limitado y determinado de personas, cumple lo previsto en la legislación sobre protección de datos de carácter personal (LOPD).

La muestra se remitirá al CBK, siguiendo las normas de conservación y transporte adecuadas, junto con el formulario de "Cesión de muestras biológicas" acompañado de una copia del consentimiento informado firmado por el donante o su representante legal. De manera opcional, se podrá cumplimentar el formulario de cesión por vía electrónica a través de la plataforma informática desarrollada por Noray Bioinformatics para el CBK, a la que se accede a través de este portal web: http://www.ciberer-biobank.es/.

El proceso de recepción de muestras se hace conforme al procedimiento interno del CBK, cumpliendo las recomendaciones y normas legales en materia de transporte de muestras biológicas.

Los productos obtenidos a partir de las muestras cedidas se almacenan y quedan custodiados en las instalaciones del CBK, atendiendo a estrictas normas de seguridad biológica aplicables tanto al procesamiento de las muestras como a su archivo y posterior envío a los grupos de investigación solicitantes.

Las muestras son almacenadas siguiendo protocolos estandarizados y su trazabilidad queda garantizada en todo momento mediante la aplicación de la plataforma informatizada diseñada específicamente para tal fin (LIMS).

La información registrada en el formulario de cesión de muestras queda almacenada, en soporte informático y en papel cumpliendo todos los requisitos establecidos por la legislación vigente.

Además, y debido a que el CBK está integrado dentro de la estructura de investigación del CIBERER, cabe destacar que el personal sanitario que remite una muestra al CBK se puede beneficiar de un servicio de información, mediante el cual se establece una relación de interés mutuo, cuyos últimos beneficiarios son los pacientes. Gracias a este servicio, el facultativo remitente tiene la opción de recibir datos actualizados en relación a la enfermedad de que se trate, a través de las bases de datos del CIBERER y Orphanet (base de datos europea de referencia de información sobre ER), cuyo socio español es CIBERER desde abril de 2010. Se facilitará, asimismo, información acerca de investigadores del CIBERER que estén trabajando en esa patología.

Simultáneamente se difunde la disponibilidad de la muestra a través del portal web del CBK para que la comunidad científica internacional y los investigadores CIBERER tengan conocimiento de ella y acceso a la misma.

El catálogo de muestras del CBK está integrado por muestras captadas prospectivamente

por el propio biobanco así como por muestras recogidas (y procesadas) por investigadores pertenecientes a algún grupo del CIBERER que cumplen los criterios de inclusión determinados por el biobanco.

Las colecciones del CBK, a disposición de aquellos investigadores que las soliciten, incluyen diferentes tipos de muestras (ADN de sangre y tejidos, PBMCs, fibroblastos, plasma, ARN, células inmortalizadas, orina, etc.), tanto de pacientes adultos como pediátricos y de diferentes partes de la geografía española, pertenecientes a más de 45 diagnósticos clínicos diferentes, tales como el síndrome de Rett, la enfermedad de Charcot-Marie-Tooth, la esclerosis tuberosa, la mielopatía degenerativa crónica, el síndrome de Sotos y la anemia de Fanconi.

Cualquier investigador que desee solicitar una muestra biológica al CBK deberá rellenar un formulario de solicitud, adjuntando un informe sobre el proyecto de investigación para el cual se requiere, así como todos aquellos datos pertinentes para la evaluación de su viabilidad (recursos humanos y financieros, infraestructura, etc.). El Comité de Ética y el Comité Científico procederán a evaluar la idoneidad de la donación de la muestra biológica solicitada y emitirán un informe al respecto. Si la decisión es favorable, la muestra será enviada al solicitante, después de que el investigador responsable se comprometa a cumplir con las condiciones establecidas por la legislación vigente relativa a la Protección de Datos y la Ley 14/2007 de Investigación Biomédica. El compromiso hace referencia al uso apropiado de las muestras, dado que constituye un material de investigación escaso de elevado valor científico, y la confidencialidad de la información asociada o cualquier otra que pudiera resultar de su estudio.

Al margen de la actividad propia de biobanco, el CBK participa en diferentes proyectos de investigación, tanto nacionales (proyectos intramurales con otros grupos de investigación

CIBERER y proyectos financiados mediante convocatorias públicas) como internacionales (RD-Connect, un proyecto financiado por el Séptimo Programa Marco).

Además, el CBK colabora con otros biobancos y redes de biobancos, tales como el Banco Nacional de ADN, la Red Valenciana de Biobancos, el biobanco del CSISP de la Generalitat Valenciana, el Biobanking and Biomolecular Resources Research Infrastructure (BBMRI) y la Red Nacional de Biobancos del Instituto de Salud Carlos III.

En resumen, el CBK es una plataforma de apoyo a la investigación que actúa como punto de encuentro entre donantes, facultativos e investigadores, con el objetivo de garantizar un tratamiento seguro y eficaz de las muestras biológicas y sus datos asociados. Su propósito es promover y facilitar los estudios en diferentes áreas de la investigación biomédica de vital importancia para el desarrollo de la medicina personalizada. Es, por tanto, una plataforma que debe ser fomentada y recibir pleno apoyo por parte de las instituciones financieras, facultativos, investigadores y pacientes, con el fin de que la investigación relacionada con las ER avance y ofrezca aquellas soluciones diagnósticas y terapéuticas que los afectados por estas patologías requieren.

UNIDAD DE ATENCIÓN A PACIENTES ADULTOS CON ERRORES CONGÉNITOS DEL METABOLISMO

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En la atención asistencial de cualquier proceso crónico que se inicia en la edad infantil debe tenerse en cuenta su seguimiento durante la edad adulta. Sin embargo, este *continuum* no siempre se lleva a cabo de una forma satisfactoria para los afectados, y ello es más evidente en el caso de las enfermedades minoritarias (EM) por su baja prevalencia y porque la experiencia en algunos tipos de procesos, en el ámbito de la asistencia a pacientes adultos, es escasa.

Los errores congénitos del metabolismo (ECM) constituyen un grupo de enfermedades cuyo diagnóstico se establece mayoritariamente en la edad infantil y su seguimiento es responsabilidad de los pediatras. Los avances en el diagnóstico precoz han permitido establecer conductas y tratamientos adecuados y al alcanzar la edad adulta estos pacientes pueden llegar a sentir una grave sensación de abandono por la inexperiencia de los facultativos en este tipo de procesos.

Por este motivo, y a iniciativa del Hospital pediátrico Sant Joan de Déu de Barcelona, de la Sección de ECM del Hospital Clínic de Barcelona (HCB) y del Servicio de Medicina Interna del HCB, con el soporte del CIBER en Enfermedades Raras (CIBERER), se constituyó hace 3 años la Unidad de atención a pacientes adultos con ECM.

Esta Unidad está constituida por un equipo asistencial multidisciplinar con el fin de atender a pacientes con dichas enfermedades y forma parte del Grupo de Trabajo de atención a pacientes adultos con EM del HCB. Este Grupo coordina 41 unidades específicamente orientadas a la atención a pacientes con EM y pertenecen a distintas especialidades.

El objetivo de la Unidad de pacientes adultos con ECM es contribuir al desarrollo de actividades asistenciales, de investigación y de formación, con el resultado de mejoras en la calidad, seguridad clínica y la eficiencia de tales enfermedades.

La Unidad de ECM está formada por médicos especialistas de Medicina Interna, Neurología, Ginecología, Endocrinología, Nutrición/Dietética y Genética Médica, además de miembros de la Sección de ECM del Centro de Diagnóstico Biológico del HCB y de investigadores del CIBERER y del Instituto de Investigación Biomédica August Pi i Sunyer (IDIBAPS). En su completo desarrollo se prevé la incorporación de profesionales de enfermería y de los Servicios Sociales.

El proyecto se inició con la derivación ordenada y progresiva desde el HSJD al HCB de todos los pacientes con distintos procesos metabólicos que habían alcanzado la edad adulta. A ellos se sumaron los pacientes adultos con miopatías

metabólicas (fundamentalmente glucogenosis tipo II y V) y otros procesos (distrofias musculares, encefalomiopatías mitocondriales, miopatías congénitas, miopatías inflamatorias) que ya estaban siendo controlados por el Servicio de Medicina Interna del HCB. Así mismo, desde su inicio se desarrolló un plan de información a los profesionales de los servicios de Farmacia y de Urgencias acerca de las necesidades de medicación específica.

Otras acciones que se están llevando a cabo o están previstas en el futuro son:

1) Consolidar las relaciones con organizaciones de pacientes.

2) Avanzar en la obtención y desarrollo de proyectos de investigación, en coordinación con el CIBERER y el IDIBAPS.

3) Consolidar un programa anual científico-docente.

4) Colaborar en el plan de unidades expertas en EM del Departament de Salut de la Generalitat de Catalunya.

5) Incentivar la formación de profesionales jóvenes interesados en ECM (y en otras EM).

6) Desarrollar la colaboración con otros grupos asistenciales con experiencia en EM nacionales e internacionales.

7) Colaborar en el registro nacional e internacional de EM.

Bortezomib in the rapid reduction of high sustained antibody titers in disorders treated with therapeutic protein: lessons learned from Pompe disease

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Bortezomib in the rapid reduction of high sustained antibody titers in disorders treated with therapeutic protein: lessons learned from Pompe disease

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Purpose: High sustained antibody titers complicate many disorders treated with a therapeutic protein, including those treated with enzyme replacement therapy, such as Pompe disease. Although enzyme replacement therapy with alglucosidase alfa (Myozyme) in Pompe disease has improved the prognosis of this otherwise lethal disorder, patients who develop high sustained antibody titers to alglucosidase alfa enter a prolonged phase of clinical decline resulting in death despite continued enzyme replacement therapy. Clinically effective immune-tolerance induction strategies have yet to be described in the setting of an entrenched immune response characterized by high sustained antibody titers, wherein antibody-producing plasma cells play an especially prominent role.

Methods: We treated three patients with infantile Pompe disease experiencing marked clinical decline due to high sustained antibody titers. To target the plasma cell source of high sustained antibody titers, a regimen based on bortezomib (Velcade) was used in combination with rituximab, methotrexate, and intravenous immunoglobulin.

Results: The treatment regimen was well tolerated, with no obvious side effects. Patient 1 had a 2,048-fold, and patients 2 and 3 each had a 64-fold, reduction in anti-alglucosidase alfa antibody titer, with concomitant sustained clinical improvement.

Conclusion: The addition of bortezomib to immunomodulatory regimens is an effective and safe treatment strategy in infantile Pompe disease, with potentially broader clinical implications.

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Key Words: antibodies; bortezomib; enzyme replacement therapy; glycogen storage disease type II; immune modulation

BACKGROUND

Protein replacement therapies have prolonged the survival and improved clinical outcomes of patients suffering from a multitude of disorders. However, therapeutic proteins are potentially immunogenic, eliciting antibody responses that reduce efficacy.[1–5] Infantile Pompe disease (IPD) results from the deficiency of lysosomal acid α-glucosidase (GAA). Classic IPD is characterized by cardiomyopathy, hypotonia, respiratory insufficiency, and, if untreated, death before 2 years of age.[6–8] Atypical, or nonclassic, patients with IPD present in infancy and typically do not have severe cardiomyopathy; and in some instances there is no cardiac involvement. Untreated patients with atypical IPD also have rapid disease progression, becoming wheelchair bound and/or ventilator dependent in the first few years of life.[9] The availability of enzyme replacement therapy (ERT) with Chinese hamster ovary cell-line-derived recombinant human acid α-glucosidase (rhGAA, alglucosidase alfa, Myozyme, Genzyme, Cambridge, MA) has led to significant improvements in overall survival and other clinical outcomes.[10–12] However, complications from the immune response to ERT are an ongoing challenge in Pompe disease. Prior studies have demonstrated the negative impact of high sustained anti-rhGAA IgG antibody titers (high sustained antibody titers; HSATs) on clinical outcomes in IPD.[2–4] More recently, there are reports of the negative impact of HSATs in adults with late-onset Pompe disease.[13,14].

Some of the patients treated with alglucosidase alfa also develop IgE antibodies and appear to be at a higher risk for anaphylaxis and severe allergic reactions.[15] IgE is measured in the setting of a hypersensitivity reaction and is not routinely tested. The longer term therapeutic efficacy of alglucosidase alfa appears to be more strongly associated with anti-rhGAA IgG antibodies. The impact of IgE antibodies on clinical outcomes, in the absence of anaphylaxis, is still unclear and requires further investigation. In addition to the antibody response, ERT has also been shown to induce a T-cell response.[16]

IPD serves as an excellent model with which to evaluate immune tolerance induction (ITI) protocols as it is a rapidly progressive disease and any clinical interventions or factors altering

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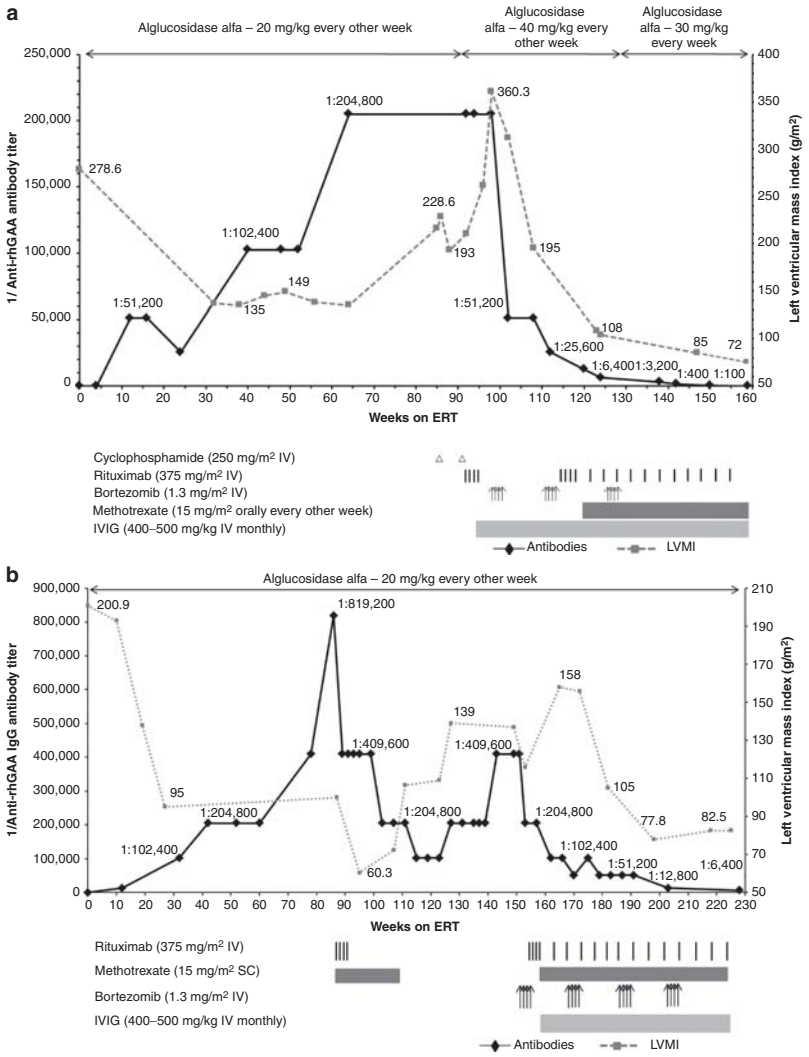
efficacy of ERT tend to manifest rapidly via well-defined clinical end points. Recently, emerging clinical evidence regarding the effect of the immune response to ERT in IPD has spawned investigations into new approaches toward ITI designed both to preclude an immune response in the ERT-naive setting and to eliminate the immune response in patients with IPD who had only recently commenced ERT.[17,18] Despite attempts at ITI in patients with IPD who developed HSAT, success has been elusive.[19,20] Combinations of various drugs, such as rituximab, cyclophosphamide, and intravenous immunoglobulin (IVIG), and plasmapheresis in IPD with HSAT and in hemophilia have shown little success in reducing HSAT.[19–23] Failure of these agents to reduce and sustain a reduced antibody titer can potentially be explained by the hitherto use of agents that do not target antibody-producing plasma cells.[20,24] Bortezomib is a proteasome inhibitor that targets both short- and long-lived plasma cells, and is an agent of potential benefit in eliminating HSAT.[25,26]

Herein we present three patients with IPD treated with ERT who, despite initial clinical improvement, subsequently declined following the development of HSAT. All three patients were treated with a bortezomib-based immunomodulatory regimen.

METHODS

We included patients who met criteria for IPD (classic or atypical) with anti-rhGAA IgG antibody titers of ≥1:51,200 on two different occasions at/or beyond 6 months post-ERT initiation.



Patients 1 and 2 met the criteria for classic IPD: ≤1% of normal GAA activity (in skin fibroblasts and/or muscle biopsy), cardiomyopathy (left-ventricular mass index (LVMI) ≥65 g/m^2 by echocardiogram), and presentation within the first year of life. Patient 3 had atypical IPD without cardiomyopathy and presented in the first year of life. Cross-reactive immunologic material (CRIM) status was determined as described earlier based on the reactivity of a pool of monoclonal and polyclonal anti-GAA antibodies capable of recognizing both native and recombinant GAA.[27,28] A patient was designated as CRIM-positive if any of the GAA protein forms (unprocessed precursor band at 110 kDa or any of the processed forms) were detectable on western blot analysis; a patient was designated as CRIM negative if none of these protein forms was detectable on western blots (processed and unprocessed).[3] Anti-rhGAA antibody titers were serially evaluated by Genzyme, as recommended in the Myozyme package insert.[29] The fold reduction in antibody titer was calculated by dividing the initial titer value by the current antibody titer value. Urinary glucose tetrasaccharide (Glc_4), a biomarker for overall glycogen burden in skeletal muscle, was determined by high-pressure liquid chromatography with UV detector and tandem mass spectrometry (electrospray ionization mass spectrometry), as previously described.[30]. After discussion with the Northern California Regional Kaiser Genetic Diseases Treatment Advisory Board, and after obtaining written parental informed consent, a trial of a bortezomib-based regimen was commenced in patient 1. The parents of patients 2 and 3 agreed to physician-directed use of bortezomib as a life-saving measure. Bortezomib was available in both hospitals' formulary for pediatric use in refractory leukemia. The bortezomib-based regimen is outlined in **Figure 1a–c** for patients 1, 2, and 3, respectively.

Case description and findings

Patient 1. This patient, a 4-year-old Caucasian male, was diagnosed with IPD at the age of 5 months. Patient demographics, CRIM status, and mutation data are shown in **Table 1**. Disease-associated signs and symptoms were first noted at age 3 months and included hypotonia as well as feeding and sucking difficulties. He had significant cardiomegaly at 5 months. Baseline (i.e., before initiation of ERT) clinical parameters, including cardiac, motor, respiratory, and feeding status, and laboratory

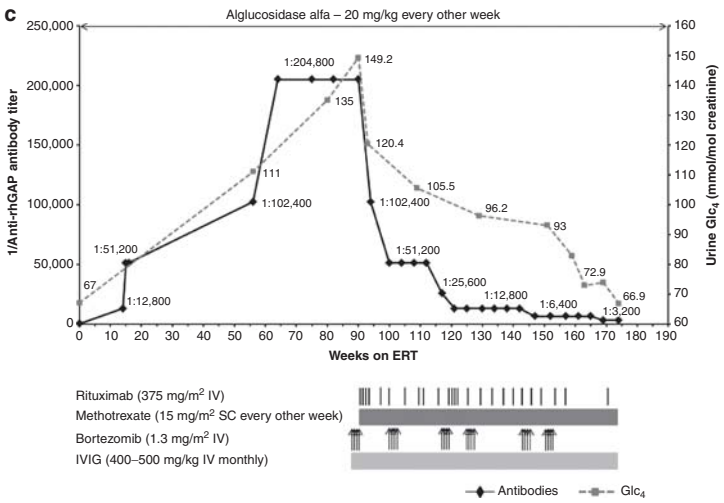


Figure 1 Trends in anti–recombinant human alglucosidase alfa (anti-rhGAA) IgG antibody titers and left-ventricular mass index (LVMI; (a and b) or urine Glc_4 (c) over time are shown by a solid black line and a dashed gray line, respectively (the upper limit of normal LVMI is 65 g/m^2;[39] control range of urine Glc_4 for age >3 years is 3 mmol/mol of creatinine). (**a**) In patient 1, following unsuccessful immunomodulation with two doses of cyclophosphamide (250 mg/m^2 i.v.) and four weekly doses of rituximab (375 mg/m^2 per dose i.v.) during weeks 86 to 95, monotherapy with bortezomib (Velcade) was initiated. Bortezomib was administered twice weekly (1.3 mg/m^2 of body surface area i.v.) according to a standard dosing regimen (days 1, 4, 8, and 11; equivalent to one cycle of bortezomib)[29] during weeks 99 and 100 (cycle 1), weeks 110 and 111 (cycle 2), and weeks 127 and 128 (cycle 3) of enzyme replacement therapy (ERT). Monthly intravenous immunoglobulin (IVIG) was administered at the start of the first cycle of bortezomib. Following a second cycle of bortezomib with continued ERT, a total of four weekly doses of i.v. rituximab (375 mg/m^2 per dose) were administered in addition to biweekly methotrexate (15 mg/m^2) orally. Rituximab was thereafter administered on a monthly basis. (**b**) In patient 2, following unsuccessful immunomodulation with rituximab (375 mg/m^2, i.v.) and methotrexate (15 mg/m^2 s.c.), bortezomib was administered as described in patient 1. Following the first cycle of bortezomib, a total of four weekly doses of rituximab (375 mg/m^2 per dose, i.v.) were administered in addition to biweekly methotrexate (15 mg/m^2, s.c.). Rituximab was thereafter administered on a monthly basis. Monthly IVIG was administered as described for patient 1. (**c**) In patient 3, bortezomib was administered as described for patient 1. Six such cycles were administered between weeks 88 and 153. Rituximab was administered (375 mg/m^2 per dose, i.v.) approximately every month except after the first and third cycle of bortezomib, at which time a total of four weekly doses were administered after the last bortezomib injection. Methotrexate and IVIG were administered as described in patients 1 and 2.

Table 1 Patient demographics, cross-reactive immunologic material status, and mutation data

		Patient 1	Patient 2	Patient 3
Gender		Male	Male	Male
Race		Caucasian	Caucasian	Caucasian
CRIM status		Positive	Negative	Positive
Mutations	Allele 1	c.307T>G	c.2560C>T	c.1655T>C
	Allele 2	c.2481+102 _2646+31del	c.1654delC	c.1655T>C
Age at symptom onset		3 months	2 months	<1 month
Age at diagnosis		5.0 months	4.0 months	3.5 years
Age at ERT start		6.0 months	4.5 months	3.6 years

CRIM, cross-reactive immunologic material; ERT, enzyme replacement therapy.

parameters such as antibody titers and urinary Glc4 levels are shown in **Tables 2–4**. At age 6 months, the patient was started on ERT with alglucosidase alfa at 20 mg/kg every other week. Clinical improvement was noted shortly after the initiation of ERT, including improvements in cardiac and motor status (**Tables 2–4**).

Rising antibody titers. From a seronegative status at week 4 of ERT, anti-rhGAA antibody titers continued to rise, peaking at 1:204,800 at week 64 of ERT, and were maintained at 1:204,800 through week 86. Concomitant with rising antibody titers was a progressive decline in clinical status and increase in urine Glc_4 levels (**Tables 2–4**). The patient became fully ventilator dependent and required tube feeds. Anti-rhGAA IgG antibody titers and LVMI values over time are shown in **Figure 1a**.

Immunomodulation with cyclophosphamide and rituximab. Due to HSATs and concurrent clinical decline, ITI was initiated. Cyclophosphamide (250 mg/m^2 i.v.) monotherapy was administered at weeks 86 and 92 of ERT, followed by rituximab (375 mg/m^2 i.v.) every week from week 92 to week 95 post-ERT initiation (**Figure 1**), based on published reports.[16,17] Despite elimination of a subset of B cells from peripheral blood, as evidenced by a CD19 count of 0%, antibody titers remained persistently high at 1:204,800, and the patient continued to decline clinically in terms of cardiac, motor, and respiratory status (**Tables 2–4**). Increasing LVMI resulted in a near-complete left-ventricular cavity obstruction and significant left-ventricular outflow tract obstruction. At this time, he also had an increase in ventilation requirement. Urinary Glc_4 levels peaked at 153.3 mmol/mol of creatinine. The patient was unable to move his arms or legs and his voluntary motor activity was limited to ocular movements.

Immunomodulation using bortezomib-based regimen and subsequent clinical improvement. Given the dire prognosis of this child, and the reasoning that rituximab-resistant, antibody-secreting plasma cells had to be eliminated, a trial of the bortezomib-based regimen was commenced (**Figure 1a**).

Following the first cycle of IV bortezomib monotherapy, antibody titers decreased from 1:204,800 to 1:51,200 (**Figure 1a**). Cycle two of bortezomib was combined with rituximab, methotrexate, and IVIG to preclude activation of rhGAA-specific naive and memory B- and T-cells and facilitate tolerance induction. This treatment resulted in a further decline of the antibody titer to 1:6,400 (**Figure 1a**). Following a third round of bortezomib in combination with rituximab and methotrexate, antibody titers were 1:100 at week 159 of ERT. The decline in antibody titers in this bortezomib-treated patient over time is in stark contrast to the persistence of HSATs in CRIM-negative and high-titer CRIM-positive patients, as well as in patients treated with a non-bortezomib-based immunomodulatory regimen (**Supplementary Figure S1** online).[19]

The reduction in antibody titers following implementation of an immunomodulation strategy using bortezomib was in close temporal association with a period of marked and prolonged clinical improvement, including significant improvement in cardiac status, decreased ventilatory requirements, and improved motor status (**Tables 2–4**). The treatment regimen was well tolerated with no apparent side effects. At the time of this report, the patient is no longer receiving bortezomib. Rituximab dose intervals have been increased to 2 months. He continues on methotrexate and IVIG, the latter as clinically needed. The patient continues to receive alglucosidase alfa infusions at 30 mg/kg every week.

Patient 2. Patient 2 is a 4½-year-old male diagnosed with IPD at 4 months of age. Patient 2 demographics, CRIM status, and mutation data are shown in **Table 1**. This patient presented with a cardiac murmur at age 2 months and an echocardiogram showed significant biventricular hypertrophy. Physical exam at age 3 months showed signs of IPD (**Tables 2–4**). At age 4.5 months, he was started on alglucosidase alfa at 20 mg/kg every other week. Clinical improvement was observed after ERT initiation and included a reduction in LVMI and improved feeding (**Tables 2–4**).

Rising antibody titers. From a seronegative status at baseline antibody titers continued to rise and peaked at 1:819,200 at week 86 of ERT. Concomitant with rising antibody titers was a plateau of his gross developmental progress and increased urinary Glc_4 (**Tables 2–4**).

Immunomodulation with rituximab and methotrexate. Given the rise in antibody titers, plateau in clinical response and his CRIM-negative status, an ITI protocol was initiated at week 87 that included rituximab (375 mg/m^2 i.v., every week for 4 weeks and methotrexate (15 mg/m^2 s.c.) every other week. This regimen led to an eightfold decrease in antibody titers over 29 weeks: 1:819,200 at week 86 to 1:102,400 at week 115. He maintained adequate physical activity and initially progressed in developmental motor milestones. Although there was an initial phase of improved LVMI, the values continued to deteriorate thereafter (**Figure 1b**, **Tables 2–4**). Even after his

Table 2 Clinical and laboratory parameters over time for the three patients treated with bortezomib-based regimen (patient 1)

Patient 1

	Baseline	Initial improvement phase	Period of rising anti-rhGAA IgG titers	Immunomodulation attempt with cyclophosphamide and rituximab	Immunomodulation with bortezomib-based regimen
	Prior to ERT	Week 1 → week 38 of ERT	Week 38 → week 86 of ERT	Week 86 → week 98 of ERT	Week 98 of ERT onward
Anti-rhGAA IgG titers	Seronegative	1:25,600 → 1:102,400	1:102,400 → 1:204,800	1:204,800	1:204,800 → 1:100
Cardiac status (LVMI (g/m^2))[a]	278.6	135	138.0 → 228.6	228.6 → 360.3 (With significant LVOTO; peak velocity 5.3 m/s)	360.3 → 72.0 (With no evidence of LVOTO)
Urinary Glc_4 (mmol/mol creatinine)	39.9[b]	NA	130.4[c] (Week 64)	153.3[c] (Week 92)	83.4[d] (Week 159)
Gross motor status	Generalized hypotonia	Sat unsupported; rolled over; raised arms against gravity; bore weight on lower extremities	Regression of motor milestones; myopathic facies	Voluntary motor activity limited to ocular movements only	Reacquisition of previously lost motor milestones; regained ability to move fingers and toes, bend knees and nod head; recovery of muscles of facial expression and improvement in speech function
Respiratory status	No respiratory support	No respiratory support	Full-time ventilator dependent (PC/PEEP of 18/6 cm H_2O, breathing rate: 6/min) (week 55 onward)	Increase in ventilation requirement (PC/PEEP of 15/6 cm H_2O, breathing rate increased to 30/min)	Decrease in ventilation requirement (PC/PEEP of 15/5 cm H_2O, breathing rate 10/min); ability to come off ventilator frequently for short periods (~8–10 min) (week 159)
Feeding status	Oral	Oral	Gastrostomy tube (week 55 onward)	Gastrostomy tube	Gastrostomy tube

Baseline: before initiation of ERT.
ERT, enzyme replacement therapy; GAA, glucosidase α; LVMI, left-ventricular mass index; LVOTO, left-ventricular outflow tract obstruction; NA, not available; PC/PEEP, pressure control/positive end-expiratory pressure; urinary Glc_4, urinary glucose tetrasaccharide.
[a]Upper limit of normal LVMI: 65 g/m^2 (>2 SD higher than upper limit of the age-appropriate normal mean). [b]Control value for ages 1 to 6 months: 20 mmol/mol of creatinine (95th percentile). [c]Control value for ages 1 to 3 years: 8 mmol/mol of creatinine. [d]Control range for ages >3 years: 3 mmol/mol of creatinine.

B-lymphocyte CD19 count dropped from 17.9% at the onset of rituximab and methotrexate therapy at week 87 to 0.1% at week 89, antibody titers did not drop to <1:102,400. The antibody titers continued to rise again and remained at peak levels of 1:409,600 from week 143 through week 153.

At age 3 years and 2 months (week 146 of ERT; ~13 months after the last dose of rituximab and 9 months after the last dose of methotrexate), he developed an upper respiratory tract infection, which led to hospitalization and intubation. During his 6-week hospitalization, he was unable to be weaned from the ventilator; he required ventilator support at discharge (**Tables 2–4**). His cardiac, motor, and feeding status deteriorated while he was hospitalized, which was concurrent with increased urinary Glc_4 levels (**Tables 2–4**).

Immunomodulation using bortezomib-based regimen and subsequent clinical improvement. A trial of bortezomib was commenced based on the following evidence: results of case 1, worsening clinical status, lack of reduction of antibody titers below 1:102,400 with the rituximab and methotrexate regimen, and the known fact of poor outcome in patients with HSATs. Details of the bortezomib-based immunomodulatory strategy are described in **Figure 1b**. Following four cycles of bortezomib, in combination with rituximab, methotrexate, and IVIG, antibody titers decreased from 1:409,600 to 1:6,400. As in patient 1, the decline in antibody titers in this bortezomib-treated patient over time is in stark contrast to the persistence of HSATs in CRIM-negative patients (**Supplementary Figure S1** online). Likewise, the observed reduction in antibody titers, from 1:409,600 to 1:6,400, was closely associated in time with clinical improvement, including cardiac, motor, respiratory, and feeding status (**Tables 2–4**). He has had no hospitalizations or illnesses since the start of the bortezomib-based immunomodulatory regimen and has tolerated this regimen well, with no apparent side effects. The patient continues to receive alglucosidase alfa infusions at 20 mg/kg every other week.

Patient 3. Patient 3 is a 6-year and 10-month-old Caucasian male diagnosed with atypical IPD at age 3.5 years based on reduced GAA activity in muscle as well as extensive cellular vacuolization with glycogen accumulation. Patient 3 demographics, CRIM status, and mutation data are shown in **Table 1**. Symptoms began in the newborn period and included the findings shown in **Tables 2–4**. Echocardiogram and electrocardiogram findings remained within normal limits, confirming

Table 3 Clinical and laboratory parameters over time for the three patients treated with bortezomib-based regimen (patient 2)

Patient 2

	Baseline	Initial improvement phase	Period of rising anti-rhGAA IgG titers	Immunomodulation attempt with cyclophosphamide and rituximab	Immunomodulation with bortezomib-based regimen
	Prior to ERT	Week 1 → week 38 of ERT	Week 38 → week 86 of ERT	Week 86 → week 98 of ERT	Week 98 of ERT onward
Anti-rhGAA IgG titers	Seronegative	1:12,800	1:102,400 → 1:819,200	1:819,200 → 1:102,400 → 1:204,800	1:204,800 → 1:6,400
Cardiac status (LVMI (g/m^2))[a]	200.9	95	95.0 → 100.0	100 → 60.3 → 139.0	139 → 82.5
Urinary Glc_4 (mmol/mol creatinine)	NA	NA	16.8[b] → 23.2[b]	23.2[b] → NA → 95.3[b]	95.3[b] → 73.9[c]
Gross motor status	Generalized hypotonia; head lag; facial myopathy; bilateral ptosis; tongue protrusion	Clinical improvement from baseline	Plateau of gross developmental progress	Regression of gross motor milestones; remained wheelchair-bound; able to sit with support; unable to stand unassisted	Stands with support; increased extremity strength; able to hold arms above body; pushes against resistance
Respiratory status	No respiratory support	No respiratory support	No respiratory support	Required ventilator support (PC/PEEP of 18/8 cm H_2O, breathing rate 18/min) (week 146)	Remains ventilator dependent; tolerates trach-mask trials off the ventilator for 10–15 min twice a day; decrease in ventilation requirement (PC/PEEP of 10/5 cm H_2O, breathing rate 14/min) (week 221)
Feeding status	Oral	Oral	Oral	Nasogastric tube (week 146 onward)	Oral

Baseline: before initiation of ERT.
ERT, enzyme replacement therapy; GAA, glucosidase α; LVMI, left-ventricular mass index; NA, not available; PC/PEEP, pressure control/positive end-expiratory pressure; urinary Glc_4, urinary glucose tetrasaccharide.
[a]Upper limit of normal LVMI: 65 g/m^2 (>2 SD higher than upper limit of the age-appropriate normal mean). [b]Control value for ages 1 to 3 years: 8 mmol/mol of creatinine. [c]Control range for ages >3 years: 3 mmol/mol of creatinine.

suspected atypical IPD. ERT with alglucosidase alfa was commenced at age 3.6 years at 20 mg/kg every other week. At this time, the patient required gastrostomy-tube feeds. Urine Glc_4 level at baseline was 67 mmol/mol of creatinine (control value for age >3 years: 3 mmol/mol of creatinine). The patient showed a good clinical response to ERT within the first 6–8 months (26–34 weeks of ERT) as shown in **Tables 2–4**.

Rising antibody titers. From a seronegative status at baseline, antibody titers rose to 1:204,800 at week 64 of ERT, where they remained through week 90 (**Figure 1c**). Along with the rise in antibody titers, this patient started to decline clinically, including worsening of motor and respiratory status (**Tables 2–4**). This was simultaneous with increased urinary Glc_4 levels (**Figure 1c**, **Tables 2–4**).

Immunomodulation using bortezomib-based regimen and subsequent clinical improvement. Given the rise in antibody titers and concurrent clinical decline, as well as our acquired experience with the two cases mentioned above, a bortezomib-based immunomodulatory regimen was initiated for patient 3 at week 88 of ERT. Details of the immunomodulatory strategy using bortezomib are described in **Figure 1c**. Following the first round of bortezomib (four doses) in combination with rituximab, methotrexate, and IVIG, the antibody titer dropped from 1:204,800 to 1:51,200 (**Figure 1c**). Subsequent administration of the bortezomib-based regimen resulted in further decline in antibody titers to 1:3,200 at week 169 of ERT (**Figure 1c**). Along with the drop in antibody titer, the patient experienced significant clinical improvement. There was a substantial reduction in ventilator requirements, as well as an overall increase in strength and energy, and significantly improved motor function (**Tables 2–4**). Improvements in speech and swallowing were also noted. Urine Glc_4 levels continued to drop from a preimmunomodulatory value of 149.2 (week 88) to 66.9 mmol/mol creatinine at week 169 (**Figure 1c**). The treatment regimen was well tolerated, with no evident side effects. The patient continues to receive alglucosidase alfa infusions at 20 mg/kg every other week.

DISCUSSION

At present, there are over 75 therapeutic proteins approved by the Food and Drug Administration for life-threatening and debilitating chronic diseases, with many more under development. A principal problem precluding the full clinical benefit derived from the use of these agents pertains to the elicitation

Table 4 Clinical and laboratory parameters over time for the three patients treated with bortezomib-based regimen (patient 3)

Patient 3

	Baseline	Initial improvement phase	Period of rising anti-rhGAA IgG titers	Immunomodulation with bortezomib-based regimen
	Prior to ERT	Week 1 → week 34 of ERT	Week 34 → week 88 of ERT	Week 88 of ERT onward
Anti-rhGAA IgG titers	Seronegative	1:12,800 → 1:51,200	1:51,200 → 1:204,800	1:204,800 → 1:3,200
Cardiac status (LVMI (g/m^2))[a]	WNL	WNL	WNL	WNL
Urinary Glc_4 (mmol/mol creatinine)	67[b]	NA	111[b] → 149.2[b]	149.2 → 66.9[b]
Gross motor status	Delayed gross motor milestones, with inability to ambulate or sit unsupported	Acquired new motor milestones; ability to ride a stationary hand-pedal exercise machine up to 15 min	Less energy and less exercise tolerance than previously noted; able to stay in a stander for 25 min	Improved motor control, including holding himself upright while in a stander; able to stay in stander for up to 3 h
Respiratory status	Required continuous ventilatory support	Improvement in ventilator status (ability to spend 1 h daily off ventilator)	Increased requirement for ventilation; remained on synchronized intermittent mandatory ventilation without a backup breath rate for 1 hour; increased need for tracheostomy suction	Decrease in ventilatory requirements with ability to come off of the ventilator for short periods of time; remains on synchronized intermittent mandatory ventilation without a backup breath rate for the whole day; decreased requirement for tracheostomy suction
Feeding status	Gastrostomy tube	Gastrostomy tube	Gastrostomy tube	Oral with improvement in swallow function plus supplementary gastrostomy tube feeds

Baseline: before initiation of ERT.
ERT, enzyme replacement therapy; GAA, glucosidase α; LVMI, left-ventricular mass index; NA, not available; urinary Glc_4, urinary glucose tetrasaccharide; WNL, within normal limits.
[a]Upper limit of normal LVMI: 65 g/m^2 (>2 SD higher than upper limit of the age-appropriate normal mean). [b]Control range for ages >3 years: 3 mmol/mol of creatinine.

of immune responses, particularly when a therapeutic protein is immunologically perceived to be a foreign antigen. This represents a common challenge when considering treatment for patients with Pompe disease, mucopolysaccharidosis types I, II, and VI, Fabry disease, and hemophilias A and B.[5,24,31]

There are various factors that could explain an immune response to a therapeutic protein, but in the patients with Pompe mounting such responses to rhGAA, the major factor is the genetic status of the patient, in which severe mutations in the *GAA* gene either fail to produce any enzyme or produce a defective enzyme that fails to tolerize the patients' immune systems. Thus, to such an untolerized immune system, the full-length human GAA appears as a foreign protein, to which an immune response is mounted.[24] Other factors of importance in elicitation of immune responses to therapeutic proteins include the following: structural properties of a protein (e.g., sequence variation and glycosylation); impurities and contaminants; frequency and duration of treatment; genetic background of patients; route of administration; and other host and environmental factors.[32]

In patients who develop HSATs against ERT, not only can the prognosis be poor but sometimes it can be fatal.[3,4,31] In addition to the human toll, the use of therapeutic proteins in patients who are not responding to the treatment because of interfering antibodies can have a large economic impact. Investigation of novel immunomodulatory strategies to preclude or reverse immune responses—and to induce immune tolerance in this setting—is not only critical in terms of therapeutic effect but also for optimal use of health-care resources.

These case studies demonstrate both the safety and efficacy of bortezomib as an immunomodulatory agent in the setting of a well-established immune response to a therapeutic protein. These are the first-known reported cases where successful induction of a prolonged decline in HSATs in a disease with therapeutic protein has been achieved by the use of a proteasome inhibitor (bortezomib). These cases demonstrate a direct relationship between the antibody response (titers and duration) and clinical response. In these three cases, treatment initiation with bortezomib was rapidly followed by sustained reductions in antibodies and clinical benefit. The rapid reduction in antibody titers occurred within a few weeks of starting the bortezomib-based regimen, with titers dropping from 1:204,800 to 1:100; 1:409,600 to 1:6,400; and 1:204,600 to 1:3,200 in patients 1, 2, and 3, respectively. This represents a 2,048-fold (patient 1) and 64-fold (patients 2 and 3) decline in titers as compared with titers at the time bortezomib was initiated. Of note, the marked and sustained decrease in antibody titers was associated with significant, durable improvement across all clinical outcome measures with continued improvement at the time of publication. Clearly, the benefits have been more robust in cardiac parameters as compared with the skeletal muscle response in patients 1 and 2. This is probably due to irreversible skeletal muscle damage that has been noted in other infantile survivors, despite long-term treatment with ERT.[12,33]

Urinary Glc_4 levels correlate with overall glycogen burden and are useful for monitoring response to ERT.[3,4,30]. For all three patients, the increase in antibody titers correlated with an

increase in urinary Glc_4 levels and clinical decline. Conversely, the decrease in antibody titers associated with immunomodulation using the bortezomib-based regimen led to a reduction (albeit of variable magnitude) in urinary Glc_4 levels and clinical improvement (**Tables 2–4**).

As with any immunosuppressive therapy, vaccination response might be diminished with the described bortezomib-based regimen. Live vaccines should be avoided while on treatment and immediately following treatment. Bortezomib is associated with peripheral neuropathy, neutropenia, thrombocytopenia, and gastrointestinal and cardiac side effects. However, no side effects were evident in any of these three patients treated with the bortezomib-based regimen. Total levels of different isotypes of immunoglobulin (total IgG, IgA, IgE, IgM, and IgD) stayed within normal ranges while on this regimen, except for two instances in patient 2 and one instance in patient 1, wherein IgA levels were above normal limits (data not shown). Given the dramatic suppression of immunity, the patients were supplemented with monthly IVIG for infection prophylaxis and to potentially contribute to immunomodulation.[34]

Although the mechanism of action of bortezomib in reducing anti-rhGAA IgG antibodies is not precisely known in these cases, we speculate that it is due to proteasome inhibition in both short-lived and long-lived plasma cells. Bortezomib binds to the catalytic site of the 26S proteasome with high affinity and specificity. There are various explanations for the actions of bortezomib and other proteasome inhibitors on plasma cells: (i) plasma cells are exceptionally sensitive to proteasome inhibitors due to their excessive protein (immunoglobulin) synthesis. Blocking the proteasome rapidly induces excessive endoplasmic reticulum stress and activation of the terminal unfolded protein response with induction of the proapoptotic protein and activation of caspases, leading to apoptosis;[26] (ii) proteasome inhibition interferes with degradation of IκB (nuclear factor of κ light polypeptide gene enhancer in B-cell inhibitors) proteins, thereby blocking activation of the transcription factor NF-κB (nuclear factor κ-light-chain-enhancer of activated B-cells), which could contribute to bortezomib-induced plasma cell death;[26] and (iii) bortezomib alters the microenvironment (e.g., interleukin-5 and interleukin-6, tumor necrosis factor-α) required for plasma cell survival and could play a role in its elimination.[26] In addition to its primary action on plasma cells, bortezomib has also been found to act on both T and B cells, thereby further modulating the humoral antibody response.[35]

As a proteasome inhibitor, bortezomib may also directly act on muscle in the context of IPD. The ubiquitin-proteasome system is believed to degrade the major contractile skeletal muscle proteins and to play a critical role in muscle wasting. Muscle wasting is a prominent feature of IPD and is similar to other muscle wasting disease states, such as cancer cachexia, sepsis, diabetes, and metabolic acidosis, in which expression of the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle.[8,36] Thus, blockade of the proteasome in muscle has the potential to mitigate skeletal muscle wasting and damage, which has been demonstrated preclinically in Duchenne and Becker muscular dystrophies as well as in rat models of denervation-induced muscular atrophy.[37,38]

Recent reports have demonstrated the successful use of immunomodulatory regimens based on rituximab, methotrexate, and either with or without IVIG, in the ERT-naive or early-ERT settings.[17,18] At present, we do not have enough evidence for the use of the bortezomib-based immunomodulatory regimen described here in the ERT-naive or early-ERT settings. Conservatively, it appears that this bortezomib-based immunomodulatory regimen should be used in patients with (i) antibody titers of ≥51,200 on two or more occasions at or beyond 6 months of ERT, as they seem to be at highest risk of clinical decline following the development of HSATs[4] and/or (ii) an increasing trend or persistence of antibody titers (regardless of titer value) with associated clinical decline.

As used in these three patients, a combination of drugs that target B cells, T cells, and plasma cells is needed to achieve long-term remission of antibodies and potential induction of immune tolerance, as depicted in **Supplementary Figure S2** online. In these cases, the combination of rituximab, methotrexate, and IVIG in addition to bortezomib appeared to be both safe and efficacious, with each drug acting on a different part of the pathway leading to antibody production (**Supplementary Figure S2** online).

The immunomodulatory approach described herein represents a promising new strategy for patients with entrenched immune responses to protein replacement therapies. It may also prove successful in addressing autoimmune disorders that fail to respond to agents targeting only T and B cells, such as rituximab and methotrexate. Thus, this immunomodulatory approach should be considered for further study in a variety of settings where patient immune response is implicated in the disease pathogenesis itself or in reduced effectiveness of the respective protein therapy.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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technology transfer. This potential conflict for Duke University has been resolved through monetization. S.G.B., S.N.P., and P.S.K. are listed as inventors on a Duke University pending patent application for the work described in this article. To date, neither Duke University nor the inventors have received any money from rights associated with this pending patent. D.V. receives clinical research support from Genzyme and serves as a board member for the MPS I Registry in which honoraria are provided for attendees at meetings by the host company, Genzyme. The other authors declare no conflict of interest.

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Allosteric Regulation of Glycogen Synthase Controls Glycogen Synthesis in Muscle

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SUMMARY

Glycogen synthase (GS), a key enzyme in glycogen synthesis, is activated by the allosteric stimulator glucose-6-phosphate (G6P) and by dephosphorylation through inactivation of GS kinase-3 with insulin. The relative importance of these two regulatory mechanisms in controlling GS is not established, mainly due to the complex interplay between multiple phosphorylation sites and allosteric effectors. Here we identify a residue that plays an important role in the allosteric activation of GS by G6P. We generated knockin mice in which wild-type muscle GS was replaced by a mutant that could not be activated by G6P but could still be activated normally by dephosphorylation. We demonstrate that knockin mice expressing the G6P-insensitive mutant display an ~80% reduced muscle glycogen synthesis by insulin and markedly reduced glycogen levels. Our study provides genetic evidence that allosteric activation of GS is the primary mechanism by which insulin promotes muscle glycogen accumulation in vivo.

INTRODUCTION

Elevated blood glucose levels after a meal are rapidly returned to normal, and during starvation blood glucose is maintained only slightly below normal. Such a sophisticated control system is essential to prevent serious dysfunctions such as loss of consciousness due to hypoglycaemia and toxicity to peripheral tissues in response to persistent hyperglycaemia (Wasserman, 2009). Glucose is distributed into multiple tissues of the body where it can be oxidized to produce energy or stored as the polysaccharide glycogen. In humans, the major cellular mechanism for disposal of ingested glucose is insulin-stimulated glucose transport into skeletal muscle, with the majority of glucose that enters muscle fibers being converted to glycogen (Shulman et al., 1990). Therefore, impaired insulin-stimulated muscle glucose transport and glycogen synthesis are implicated in the pathogenesis of insulin resistance and type 2 diabetes (Shulman, 2000).

Insulin stimulates glucose transport by promoting the translocation of the glucose transporter, GLUT4, from intracellular compartments to the cell surface. The resulting increase in glucose transport and phosphorylation of glucose by hexokinase leads to an increase in the intracellular concentration of glucose-6-phosphate (G6P). G6P is mainly used for the synthesis of glycogen in resting muscle, while it is largely metabolized in the glycolytic pathway during contraction. During glycogen synthesis, G6P is converted to UDP-glucose and the glucosyl unit from UDP-glucose is used to elongate a nascent glycogen chain through α-1,4-glycosidic bonds by the action of glycogen synthase (GS), which is stimulated by insulin (Roach, 2002). This activation of GS in response to insulin occurs in minutes and was the first example of regulation of a specific enzyme by insulin in cells (Villar-Palasi and Larner, 1960). Despite decades of intensive research in this field, the molecular mechanism(s) whereby insulin activates GS in vivo is controversial, although several models have been proposed (Lawrence and Roach, 1997).

In the early 1960s two mechanisms were identified by which GS activity could be regulated: allosteric activation by G6P (Leloir et al., 1959) and covalent phosphorylation (Friedman and Larner, 1963), which inhibits the enzyme. Insulin activates GS through allosteric regulation by elevating the levels of G6P via glucose transport and also phospho-dependent mechanisms by promoting the conversion of GS from a highly phosphorylated (low-activity) form to a less phosphorylated (high-activity) form through the protein kinase B (PKB, also known as Akt)-dependent inactivation of glycogen synthase kinase 3 (GSK3) (McManus et al., 2005). Dephosphorylation results in significant changes in the kinetic properties of GS, decreasing the Km for the substrate UDP-glucose and the $A_{0.5}$ for G6P (Friedman and Larner, 1963; Jensen and Lai, 2009). Thus, it is thought that insulin stimulates GS through the complex interplay between phosphorylation and allosteric regulation. A major obstacle to gaining molecular insight into how insulin regulates GS in vivo is a lack of understanding of the relative importance of these two regulatory mechanisms, allosteric and phospho dependent, in controlling GS activity.

To understand the contribution that phospho-dependent activation of GS plays in insulin-stimulated muscle glycogen synthesis, we previously generated knockin mice expressing

constitutively active mutants of GSK3 (both α and β isoforms) in which the PKB phosphorylation sites on GSK3α (Ser21) and GSK3β (Ser9) were substituted by Ala (GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$) (McManus et al., 2005). In GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$ animals, we found that insulin failed to inactivate muscle GSK3 and hence promote GS activation by dephosphorylation. Strikingly, we found that insulin-stimulated glycogen synthesis and the levels of glycogen in skeletal muscle were similar between wild-type and GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$ mice (Bouskila et al., 2008). These observations led us to hypothesize that the allosteric activation of GS by G6P plays a major role in glycogen synthesis, which would compensate for the lack of phospho-dependent activation of GS by insulin in the GSK3$\alpha/\beta^{S21A/S21A/S9A/S9A}$ mice. While this is an attractive hypothesis, no robust cell-based assays or genetic models are available to establish the in vivo role that allosteric activation of GS plays in muscle glycogen synthesis.

To this end, we aimed to identify critical residues for the G6P-mediated allosteric activation of GS, which could be exploited to generate a knockin mouse expressing a G6P-insensitive GS mutant. Here we report genetic evidence that the allosteric activation of GS is the primary mechanism by which insulin promotes glycogen synthesis in muscle.

RESULTS

Identification of Critical Residue(s) Important for Allosteric Activation of GS by G6P

Alignment of the sequences of *Saccharomyces cerevisiae* and mammalian GS isoforms revealed that there is a highly basic segment present at the C terminus of both yeast and mammalian GS (Figure 1A). Pederson et al. performed scanning mutagenesis analysis using the major *Saccharomyces cerevisiae* GS isoform, Gsy2p, and reported that mutation of multiple arginine residues within this conserved basic segment to alanine blocked the allosteric activation by G6P (Pederson et al., 2000). They subsequently demonstrated that corresponding mutations had a similar effect on rabbit muscle GS (GYS1) (Hanashiro and Roach, 2002). However, ectopic expression of the G6P-resistant rabbit muscle GS mutant R578A/R579A/R581A (all three of the indicated Arg residues changed to Ala) in COS-1 cells revealed a reduced expression level and a markedly higher basal activity when assayed in the absence of G6P relative to the wild-type enzyme (Hanashiro and Roach, 2002). Substitution of multiple Arg residues to Ala likely disrupted the three-dimensional structure, resulting in destabilization and dysfunction of the enzyme. In the current study, our initial goal was to identify a G6P-insensitive muscle GS mutant that expressed at normal levels in cells and also displayed unaltered phospho-dependent activity. We generated a series of mutants in which individual Arg residues or a combination of two or three Arg residues in the highly conserved basic segment (Figure 1A) were changed to Ala or Glu. We transfected HEK293 cells, which express only trace amounts of endogenous GS (Figure 1B), with these GS mutants together with glycogenin, a specialized initiator protein in glycogenesis which binds to and enhances expression of soluble GS (Skurat et al., 1993). Thirty-six hours following transfection of these mutants, cell extracts were generated, and expression of GS and glycogenin, as well as GS activity, was assessed (Figure 1B). Consistent with a previous report (Hanashiro and Roach, 2002),

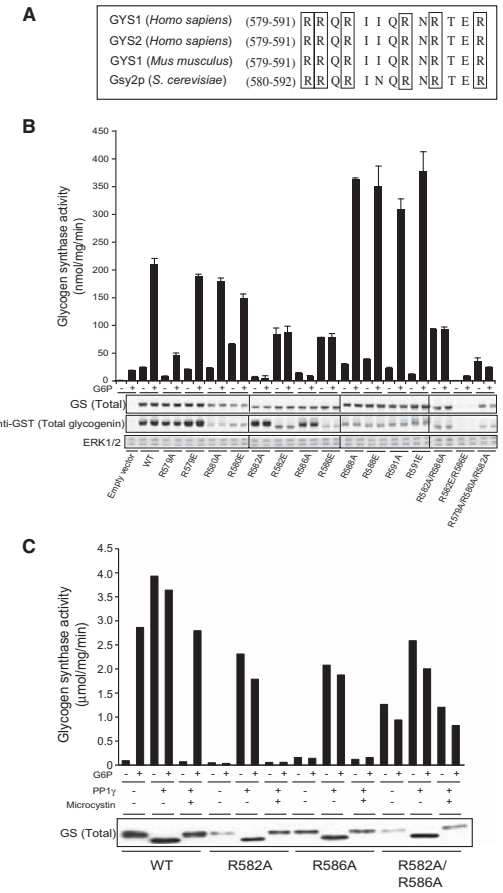

Figure 1. Mutagenesis and Biochemical Analyses to Identify Glucose-6 Phosphate-Insensitive Glycogen Synthase Mutants

(A) Amino acid sequence alignment of putative G6P sensitizing residues of mammalian and yeast GS. Arginine (R) residues located in the G6P-sensitizing region shown in (A) were mutated individually or in combination to either alanine (A) or glutamic acid (E).

(B) Constructs expressing wild-type and the indicated GS mutants were cotransfected with GST-tagged glycogenin. Cell extracts were prepared and equal amounts of protein were immunoblotted with the indicated antibodies or assayed for GS activity in the presence and absence of 10 mM G6P. The ERK1/2 kinases were immunoblotted as a loading control. Results are representative of two independent experiments.

(C) Equal quantities of purified GS mutants were dephosphorylated in vitro using PP1γ. The PP1 inhibitor microcystin-LR was used as a negative control. Mock and PP1γ-treated GS mutants were assayed for GS activity in the presence or absence of G6P and immunoblotted to confirm equal loading and assess the phosphorylation status of GS. Results are representative of two independent experiments.

we observed that the R579A/R580A/R582A triple mutant was insensitive to G6P and had modestly reduced expression compared to the wild-type enzyme. We also observed that

several other mutants including single R582A, R582E, R586A, R586E, R586A, and double R582A/R586A and R582E/R586E mutants were also resistant to G6P (Figure 1B). However, the R582E/R586E mutant displayed markedly reduced cellular expression, and single R582E, R586E, and double R582A/R586A mutants had ~3-fold higher basal activity compared to wild-type when assayed in the absence of G6P. We noticed that the expression of glycogenin was reduced (e.g., R580A, R580E, R586E) and gel mobility increased (e.g., R582E, R586E) when particular GS mutants were cotransfected (Figure 1B). We are unable to provide an accurate molecular explanation for these alterations in glycogenin. We speculate that certain amino acid substitutions may destabilize the interaction between GS and glycogenin, resulting in reduced expression of both enzymes (e.g., R580, R580E, R582E/R586E, R579A/R580A/R582A). Alternatively, amino acid substitution may have caused a change in intrinsic activity/conformational change in the GS/glycogenin complex, resulting in undefined posttranslational modifications (e.g., glycosylation, phosphorylation) of glycogenin by an unidentified mechanism(s). Among the mutants analyzed, only two single-point mutants (R582A and R586A) retained normal levels of expression (soluble) and catalytic activity (in the absence of G6P) relative to the wild-type enzyme (Figure 1B). While a trace amount of wild-type and mutant GS, as well as glycogenin, was found in the particulate fraction of cell lysates, this also occurred at similar levels (data not shown). We then examined if these point mutants (R582A and R586A) possessed normal phospho-dependent activity. Since GS proteins overexpressed in HEK293 cells were highly phosphorylated, we determined if R582A and R586A mutants could be activated to the same degree as wild-type by dephosphorylation in vitro. Purified wild-type, R582A, R586A, and R582A/R586A GS mutants were incubated in the presence of type 1 protein phosphatase (PP1) γ. Wild-type GS was efficiently dephosphorylated by PP1γ, as evidenced by faster gel mobility (Figure 1C), complete loss of immunoreactivity to a phospho-specific GS antibody (data not shown), and the fact that catalytic activity of the dephosphorylated enzyme was no longer dependent on G6P (basal activity equivalent to G6P-saturated activity) (Figure 1C). These effects were blocked by the PP1 inhibitor microcystin-LR (Figure 1C). Notably, PP1γ promoted a robust activation of G6P-resistant R582A and R586A mutants with or without G6P close to the levels observed in wild-type, which was associated with a marked dephosphorylation as judged by gel mobility shift of total GS (Figure 1C) and staining with phospho-specific antibodies (data not shown). Catalytic activity of the double R582A/R586A mutant was also increased by PP1γ-dependent dephosphorylation, although a markedly higher activity in the absence of G6P compared to wild-type was observed (Figures 1C and 1A). Taken together, we have identified key Arg residues that are essential for the allosteric activation of GS by G6P. The single residues, Arg582 and Arg586, can be substituted for alanine to generate mutant enzymes that are completely resistant to G6P yet retain normal cellular expression and phospho-dependent activity when expressed in HEK293 cells.

Generation and Characterization of G6P-Insensitive GS Knockin Mice

To explore the physiological roles that allosteric activation of GS by G6P might play in muscle glycogen metabolism in vivo, a knockin mouse was generated in which the codon for arginine 582 of *GYS1* (muscle isoform) was modified to encode alanine (Figures 2A–2C). When the litters from $GS^{+/R582A}$ breeding pairs were genotyped, we observed that $GS^{R582A/R582A}$ mice were born at a slightly lower rate (18.4%) than the expected normal Mendelian frequency (25%). Regardless, $GS^{R582A/R582A}$ knockin mice displayed no overt phenotype, and growth curves from 5 to 18 weeks of age in both males and females indicated that these animals were of normal size and weight (see Figure S1A available online). Likewise, $GS^{R582A/R582A}$ knockin mice consumed a similar amount of food compared to the wild-type (Figure S1B).

We determined if expression of mutant R582A GS in $GS^{R582A/R582A}$ animals was comparable to wild-type GS in several tissues. Immunoblot analysis revealed that there was no difference in the levels of muscle GS between wild-type and $GS^{R582A/R582A}$ knockin mice in various skeletal muscles and other tissues (Figure 2D). We also observed that >98% of GS protein in muscle lysates from wild-type and $GS^{R582A/R582A}$ knockin mice was recovered in the soluble fraction (supernatant after centrifugation at 3600 × g for 5 min) and the amount of GS protein detected in the pellet was similar between these two genotypes (data not shown). We confirmed there was no compensatory expression of the liver GS isoform in tissues of $GS^{R582A/R582A}$ mice, as it was expressed at a similar level in the liver of wild-type and $GS^{R582A/R582A}$ animals and was undetectable in muscles in both wild-type and $GS^{R582A/R582A}$ mice (Figure 2D). We next tested the effect of G6P on GS activity in wild-type, $GS^{+/R582A}$, and $GS^{R582A/R582A}$ mice. As shown in Figure 2E, although G6P robustly activated GS in muscle extracts derived from wild-type animals, there was a complete loss of GS activation by G6P at submaximal (0.3 mM) and saturating (12 mM) concentrations in extracts from $GS^{R582A/R582A}$ mice. G6P-stimulated GS activity in muscle lysates from $GS^{+/R582A}$ animals was reduced by more than 50% compared to wild-type (~75%), suggesting that the mutant enzyme exerts a dominant-negative effect on wild-type GS. The G6P-independent activity (0 mM) was comparable in all three genotypes.

Insulin Signaling and Phospho-Dependent GS Activation Are Normal in $GS^{R582A/R582A}$ Mice

We next examined the regulation of phospho-dependent GS activity by insulin in $GS^{R582A/R582A}$ knockin animals. Insulin injection robustly stimulated PKB phosphorylation and activity to the same extent in wild-type, $GS^{+/R582A}$, and $GS^{R582A/R582A}$ animals (Figures 3A and 3B). PKB reduces the activity of GSK3α/β by phosphorylation of inhibitory serine residues (S21/S9), which was induced by insulin to a similar extent across all three genotypes (Figure 3B). Using phospho-specific antibodies directed against GSK3-target sites on GS (Ser641/Ser645), we found that insulin promoted a marked dephosphorylation that was similar in the muscles of the wild-type, $GS^{+/R582A}$, and $GS^{R582A/R582A}$ animals (Figure 3D). There was no significant difference in phospho-dependent GS activity (measured in the absence of G6P) for the three genotypes, and insulin injection resulted in an ~2-fold activation (Figure 3C). These results demonstrate that insulin-mediated phospho-dependent GS activation is normally maintained in GS knockin mice.

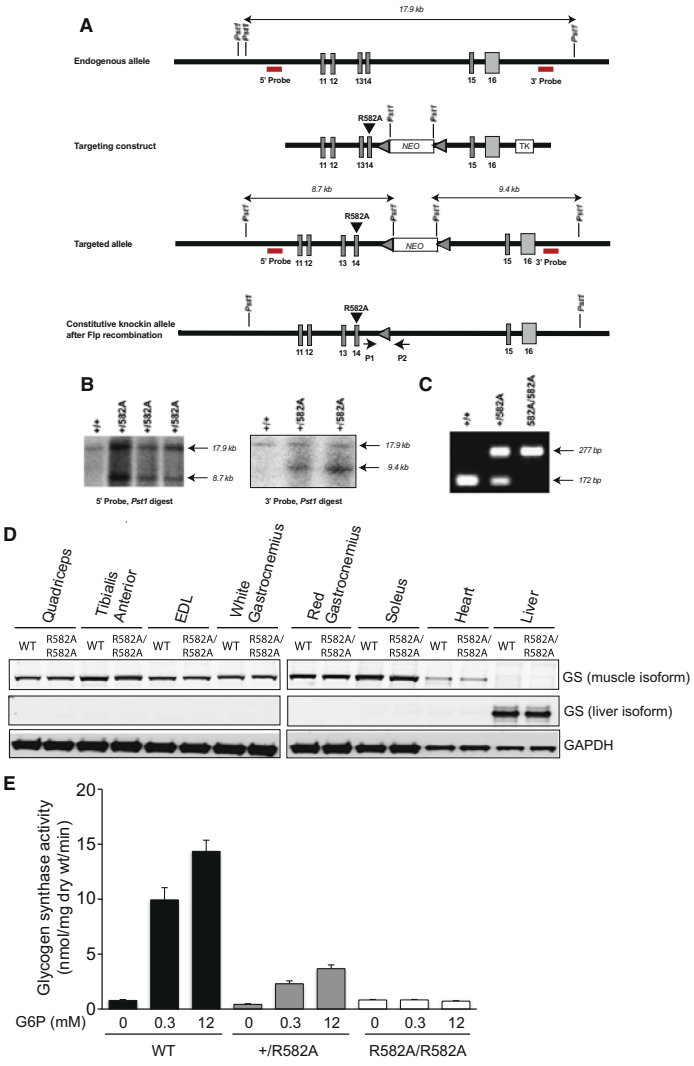

Figure 2. Targeting Strategy Employed to Generate *GYS1* R582A Knockin Mutation and Tissue Expression and Activity of GS in GS$^{R582A/R582A}$ Knockin and Wild-Type Mice

(A) Diagram showing the endogenous *GYS1* allele, the targeting knockin construct, the targeted allele with the neomycin selection cassette (NEO) still present, and the targeted allele with the NEO removed by Flp recombinase. The gray boxes represent exons (6–11), and the gray triangles represent the *FRT* sites. The knockin allele containing the Arg582Ala mutation in exon 14 is illustrated as a black rectangle.

(B) Genomic DNA isolated from the targeted embryonic stem cells from the indicated genotypes was digested with PstI and subjected to Southern blot analysis with the corresponding DNA probes (red boxes). The endogenous wild-type allele generates a 17.9 kb fragment with both 5′ and 3′ probes, while the targeted knockin allele gives rise to a 8.7 kb fragment with the 5′ probe (left panel) and a 9.4 kb fragment with the 3′ probe (right panel).

(C) Genomic DNA isolated from mouse ear biopsies was subjected to PCR analysis with the primer pairs, P1 and P2. The wild-type allele produces a 172 bp DNA fragment, while the knockin allele produces a 277 bp fragment.

(D) Equal amounts of extracts from skeletal muscle (various), heart, and liver isolated from the indicated genotypes were immunoblotted with total muscle or total liver GS isoform-specific antibodies. GAPDH was immunoblotted as a loading control. Results are representative of two independent experiments performed with tissues from four mice.

(E) Gastrocnemius muscle from the indicated genotypes was removed and freeze dried, and extracts were prepared. GS activity was measured in the absence and presence of a range of G6P concentrations. Results are representative of two independent experiments (n = 8/group).

Allosteric Activation of GS Plays a Major Role in Glycogen Synthesis In Vivo

We next sought to determine the role that allosteric activation of GS by G6P plays in muscle glycogen synthesis. The rate of glycogen synthesis in isolated skeletal muscle was measured by monitoring incorporation of [^{14}C]-glucose into endogenous glycogen particles. Soleus muscle, containing predominantly slow twitch-oxidative fibers, or extensor digitorum longus (EDL) muscle, containing predominantly fast twitch-glycolytic fibers, was isolated and incubated in the presence of 5.5 mM glucose with or without insulin. In the absence of insulin, glycogen synthesis in both muscle types was reduced by ~20%–30% in GS$^{+/R582A}$ and ~70% in the GS$^{R582A/R582A}$ mice when compared to wild-type animals (Figures 4A and 4B). Insulin stimulated glycogen synthesis by 4- and 2-fold in soleus (Figure 4A) and EDL (Figure 4B), respectively, which was reduced ~50% in GS$^{+/R582A}$ mice and by ~80% in both muscle types isolated from GS$^{R582A/R582A}$ mice compared to wild-type. We next measured glycogen concentration in muscle tissue. We found a robust decrease (~50%) and an intermediate (~20%–30%) reduction in glycogen content in muscle derived from GS$^{R582A/R582A}$ and GS$^{+/R582A}$ knockin mice, respectively, under both fasted and fed conditions (Figure 4C and Figures S2A and S2B). We observed that the levels of liver glycogen were comparable between wild-type and knockin animals in fasted and fed states (Figure 4D). In order to check if an enhanced rate of glycogen degradation contributed to the reduced muscle glycogen content observed in GS$^{+/R582A}$ and GS$^{R582A/R582A}$

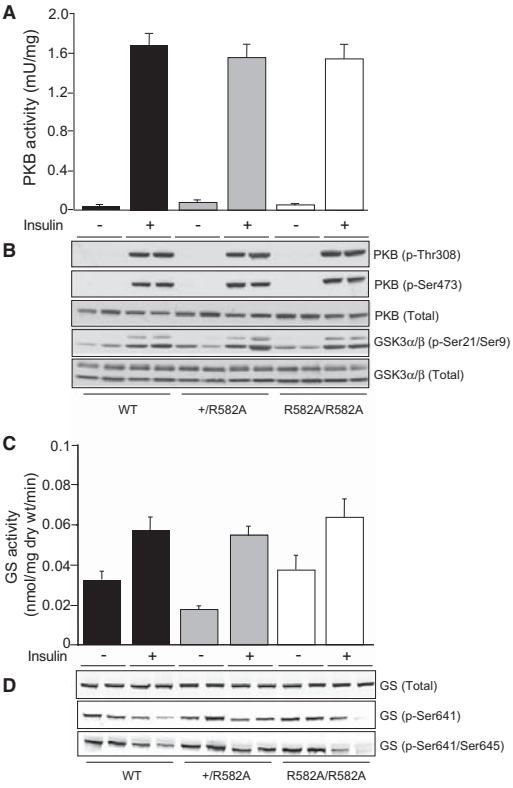

Figure 3. Analysis of Insulin Signaling and Phospho-Dependent GS Activity in GS$^{R582A/R582A}$ Mice and Wild-Type Mice

The indicated mice were fasted overnight and injected intraperitoneally with insulin or vehicle (saline) under anesthesia. After 40 min, gastrocnemius muscle was isolated, and tissue homogenates were assayed as follows.

(A) PKB kinase activity was measured in immunoprecipitates as described in the Supplemental Experimental Procedures. The assay was performed in duplicate (n = 4–6/group).

(B) PKB and GSK3α/β expression and phosphorylation were assessed by immunoblotting with the indicated antibodies. Results are representative of two independent experiments performed with tissues from four mice.

(C) GS activity in the absence of G6P was assayed. The assay was performed in duplicate and the data shown are representative of two independent experiments (n = 4–6/group).

(D) Total and phosphorylated GS were assessed by immunoblotting with the indicated antibodies.

knockin mice, we measured the activity of glycogen phosphorylase, a rate-limiting enzyme in glycogen breakdown. Phosphorylase activity in resting and insulin-stimulated muscles was similar between wild-type and GS knockin mice (Figure 4E). Consistent with this observation, phosphorylation at Ser15, a key regulatory residue, was unchanged in the different genotypes in both basal and insulin-stimulated states (Figure 4E).

To determine if the residual increase in muscle glycogen synthesis in response to insulin observed in GS$^{R582A/R582A}$ mice is due to dephosphorylation of GS via GSK3 inactivation, we have generated triple knockin mice that carry mutations in GS (GS$^{R582A/R582A}$) and GSK3α and -β (GSK3α/β$^{S21A/S21A/S9A/S9A}$). The triple knockin mice were viable, of normal size, and exhibited no overt phenotype. Immunoblot analysis showed that insulin failed to promote significant dephosphorylation of GS at Ser641 in muscles from the triple knockin mice (Figure S2E), consistent with our previous findings in GSK3α/β$^{S21A/S21A/S9A/S9A}$ mice (McManus et al., 2005). These mice displayed an additional ~10% decrease in insulin-stimulated glycogen synthesis compared to GS$^{R582A/R582A}$ mice in insulin-stimulated glycogen synthesis compared to GS$^{R582A/R582A}$ mice, although there remained a significant increase in glycogen synthesis upon insulin treatment (Figure 4F).

Reduced Glycogenic Activity Results in an Increased Glucose Flux through the Glycolytic Pathway

To investigate if the reduction in glycogen synthesis observed in muscles from GS$^{R582A/R582A}$ mice was due in part to impaired glucose transport activity, we measured 2-deoxyglucose uptake in isolated muscles ex vivo. We observed no difference in resting glucose uptake between wild-type and GS knockin animals, and insulin stimulated glucose uptake by ~4-fold and ~1.5-fold in soleus (Figure 5A) and EDL (Figure 5B), respectively, in all genotypes. Immunoblot analysis confirmed that neither GLUT1 nor GLUT4 protein levels were altered in muscles from GS$^{R582A/R582A}$ mice (Figure 5G). Resting as well as insulin-stimulated G6P levels in soleus muscles from GS$^{R582A/R582A}$ mice were higher (~2- and ~2.5-fold, respectively) than those from wild-type mice (Figure 5C). UDP-glucose levels in resting muscles were not significantly different between GS$^{R582A/R582A}$ and wild-type mice, whereas insulin robustly reduced (2-fold) UDP-glucose levels in the soleus of wild-type mice alone and was without effect on GS$^{R582A/R582A}$ animals (Figure 5D). We measured the levels of hexokinase II and UDP-glucose pyrophosphorylase in muscle extracts and confirmed that expression of both enzymes was unchanged in the muscle of GS$^{R582A/R582A}$ mice (Figure 5G). Since glycogen synthesis was profoundly reduced in the muscles of GS$^{R582A/R582A}$ animals, we hypothesized that glycolytic utilization of glucose would be enhanced in these muscles. Soleus muscle was incubated in the presence of 5.5 mM glucose containing [5-^{3}H]-glucose in KRB with or without insulin. The rate of glycolysis was monitored by measuring [^{3}H] H_2O released from the muscle into the incubation medium, and glycogen synthesis was assessed by monitoring [5-^{3}H] glucose incorporation into glycogen (Figure 5E). The rate of glycolysis in both resting and insulin-stimulated muscles was robustly increased (~2.5-fold) in GS$^{R582A/R582A}$ mice relative to that of wild-type animals. Consistent with this observation, lactate release was also higher in GS$^{R582A/R582A}$ muscle in both basal and insulin-stimulated conditions relative to wild-type muscle (Figure 5F).

GS$^{R582A/R582A}$ Mice Display Normal Whole-Body Glucose Disposal and Insulin Sensitivity

We investigated if reduced muscle glycogen synthesis and elevated intramuscular G6P levels would affect whole-body glucose homeostasis in GS$^{R582A/R582A}$ knockin mice. As illustrated in Figures 6A and 6B, there was no difference in blood glucose and plasma insulin levels from overnight fasted or

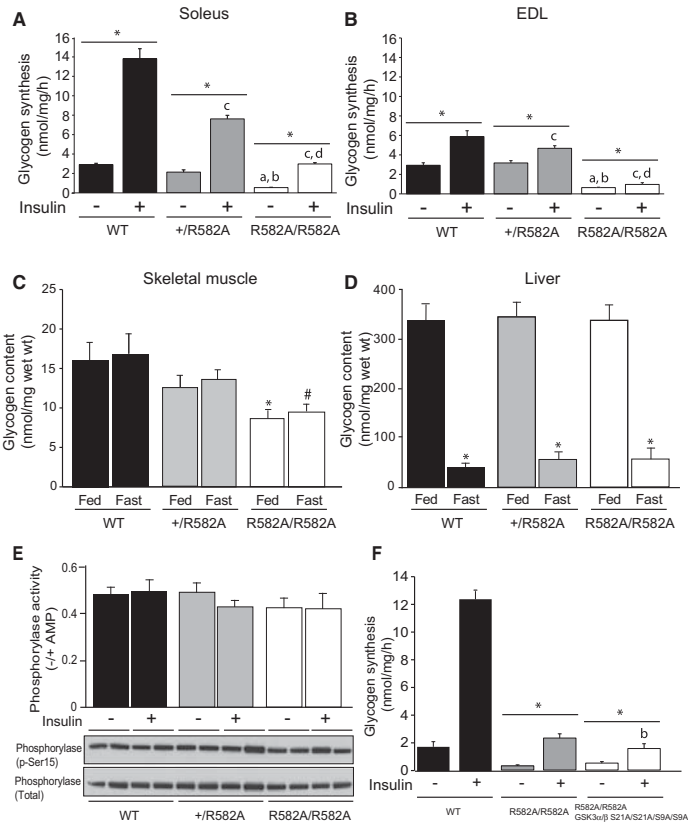

Figure 4. Analysis of Muscle Glycogen Metabolism in GS$^{R582A/R582A}$ Knockin and Wild-Type Mice

(A–D) Soleus or EDL muscles were isolated from the indicated mice following overnight fast and incubated in KRB buffer in the presence or absence of 100 nM insulin. Glycogen synthesis in soleus (A) and EDL (B) was measured as described in the Supplemental Information. *p < 0.05 basal versus insulin within each genotype; (a) p < 0.05 versus WT (basal); (b) p < 0.05 versus +/R582A (basal); (c) p < 0.05 versus WT (insulin); (d) p < 0.05 versus +/R582A (insulin) (n = 5–7/group). (C and D) The indicated mice were fasted overnight or fed ad libitum and muscle (gastrocnemius, C) and liver (D) glycogen content was assessed. *p < 0.05 WT (Fast) versus other genotypes (Fed); #p < 0.05 WT (Fed) versus other genotypes (Fed). (n = 5–7/group) (see also Figure S2).

(E) The indicated mice were fasted overnight and injected intraperitoneally with insulin or saline (basal) under anesthesia. After 40 min, gastrocnemius muscle was removed, snap frozen, and processed to generate tissue extracts. Phosphorylase activity was measured as described in the Supplemental Experimental Procedures. Expression and phosphorylation of phosphorylase were assessed by immunoblotting muscle lysates with the indicated antibodies. (n = 4–5/group).

(F) The rate of glycogen synthesis in resting and insulin-stimulated EDL muscles from the indicated genotypes was measured ex vivo. Statistical analysis (ANOVA) was performed to reveal the difference between GS$^{R582A/R582A}$ and triple knockin animals. *p < 0.05 basal versus insulin within each genotype; (b) p < 0.05 versus GS$^{R582A/R582A}$ (insulin). (n = 6–8/group).

randomly fed animals between wild-type and GS$^{R582A/R582A}$ knockin mice. We next performed glucose tolerance tests and found that GS$^{R582A/R582A}$ knockin mice were able to dispose of injected glucose at the same rate compared to their wild-type littermates in both males (Figure 6C) and females (data not shown). Similarly, insulin tolerance tests revealed an identical profile of insulin-induced hypoglycemia and recovery over the time period tested in wild-type and GS$^{R582A/R582A}$ knockin mice (Figure 6D). To further investigate the insulin sensitivity of GS$^{R582A/R582A}$ knockin mice in a more physiological context, we performed euglycaemic-hyperinsulinaemic clamps, a method that is considered the gold standard for assessing whole body glucose homeostasis in vivo. GS$^{R582A/R582A}$ knockin mice displayed no modification in whole-body glucose metabolism judged by similar rates of glucose infusion and disappearance during the insulin clamp (Figures 6E and 6F and Figure S3).

DISCUSSION

GS is an enzyme of historical importance, as it was the first example of an intracellular target for insulin (Villar-Palasi and Larner, 1960) and is also classically used as an example of enzymes that are controlled via allosteric and covalent mechanisms in cells. Kinetic properties of the differentially phosphorylated forms of GS in cell-free assays have been extensively documented since 1959 (Leloir et al., 1959; Roach, 2002), and several transgenic mouse studies suggest physiological significance of both allosteric and covalent regulation of GS in glycogen accumulation in intact skeletal muscle tissue (reviewed in Lawrence and Roach, 1997). However, the relative importance of these two regulatory mechanisms, particularly the contribution of allosteric regulation to the control of GS and glycogen storage in muscle, is not established, mainly due to the complex interplay between multiple phosphorylation sites and allosteric effectors and the absence of robust experimental tools (e.g., cell-based assays and genetic animal models). Identification of key residues involved in the allosteric regulation of GS by G6P (Figure 1 and Pederson et al., 2000) and generation of the GS$^{R582A/R582A}$ knockin mouse enabled us to overcome various inherent problems (discussed in Bouskila et al., 2008), and here we provide compelling evidence that allosteric activation of GS is the primary mechanism by which insulin promotes

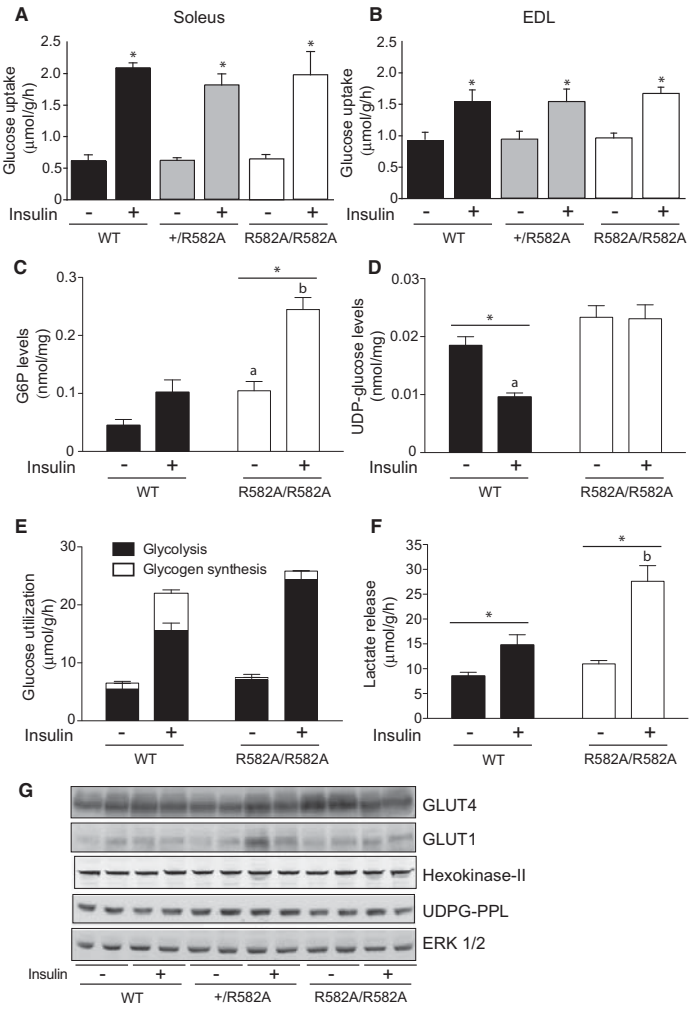

Figure 5. Analysis of Muscle Glucose Utilization in GS$^{R582A/R582A}$ Knockin and Wild-Type Mice

Soleus or EDL muscles were isolated from the indicated genotypes following overnight fast and incubated in KRB buffer in the presence or absence of 100 nM insulin.

(A and B) 2-deoxyglucose uptake in soleus (A) or EDL (B) was measured as described in the Supplemental Experimental Procedures. *p < 0.05 basal versus insulin within each genotype; (n = 5–7/group).

(C–E) Isolated soleus muscle was incubated in KRB buffer containing 5.5 mM glucose for 40 min with or without 100 nM insulin. Muscle G6P (C) and UDP-glucose (D) levels and lactate release (E) were measured as described in the Supplemental Information.

(F) Isolated soleus was incubated in KRB buffer with 5.5 mM glucose containing [5-^{3}H]-glucose for 40 min in the presence or absence of 100 nM insulin. Glucose utilization by glycolysis and glycogenesis was assessed by measuring [^{3}H]-H_2O accumulation in the incubation buffer and [^{3}H]-glucose incorporation into glycogen, respectively, as described in the Experimental Procedures. *p < 0.05 basal versus insulin within each genotype; (a) p < 0.05 versus WT (basal); (b) p < 0.05 versus WT (insulin). (n = 3–4/group).

(G) The indicated mice were fasted overnight, and gastrocnemius muscles from saline- or insulin-injected (i.p., 150 mU/g body weight, 40 min) animals were removed and tissue extracts generated. The expression of GLUT1, GLUT4, hexokinase II, and UDP-glucose pyrophosphorylase (UDPG-PPL) was assessed in muscle extracts by immunoblotting with the indicated antibodies. Four to five samples for each condition from the indicated mice were analyzed, and representative blots are shown.

glycogen synthesis upon incorporation of glucose into muscle cells. Analysis of triple GS$^{R582A/R582A}$/GSK3α/$\beta^{S21A/S21A/S9A/S9A}$ knockin mice, in which both allosteric- and phospho-dependent activation of GS by insulin is prevented, complemented the results obtained from individual GS$^{R582A/R582A}$ (current study) and GSK3α/$\beta^{S21A/S21A/S9A/S9A}$ (Bouskila et al., 2008; McManus et al., 2005) knockin mice. We observed a modest, but significant decrease (~10%) in insulin-stimulated glycogen synthesis in the muscle of the triple GS$^{R582A/R582A}$/GSK3α/$\beta^{S21A/S21A/S9A/S9A}$ knockin compared to that of GS$^{R582A/R582A}$ mice (Figure 4F). Therefore, dephosphorylation of GS as occurs with insulin is likely to play a "fine-tuning" role for GS by promoting changes in enzyme kinetic properties, which would be expected to enhance the affinity for the substrate UDP-glucose and also sensitivity to G6P (Friedman and Larner, 1963; Jensen and Lai, 2009). Whether G6P-bound/dephosphorylated GS proteins change localization for efficient glycogen formation would be an interesting subject to explore in the future. The mechanism underlying the residual increase in glycogen synthesis in response to insulin in triple GS$^{R582A/R582A}$/GSK3α/$\beta^{S21A/S21A/S9A/S9A}$ knockin mice is unclear. There was no detectable expression of the liver GS isoform (GYS2) in the muscle of GS$^{R582A/R582A}$ mice (Figure 2D), and it is unlikely that an alternative GS-like enzyme catalyzes the formation of glycogen, as muscle GS-deficient animals display a loss of glycogen (Pederson et al., 2004). Although it would be reasonable to assume that elevated UDP-glucose arising from elevated insulin-stimulated glucose uptake would increase glycogen synthesis by mass action, results from our work and others are not sufficient to draw this conclusion. An increase in the levels of G6P would be expected to increase concentrations of downstream intermediates by mass action in the glycogen

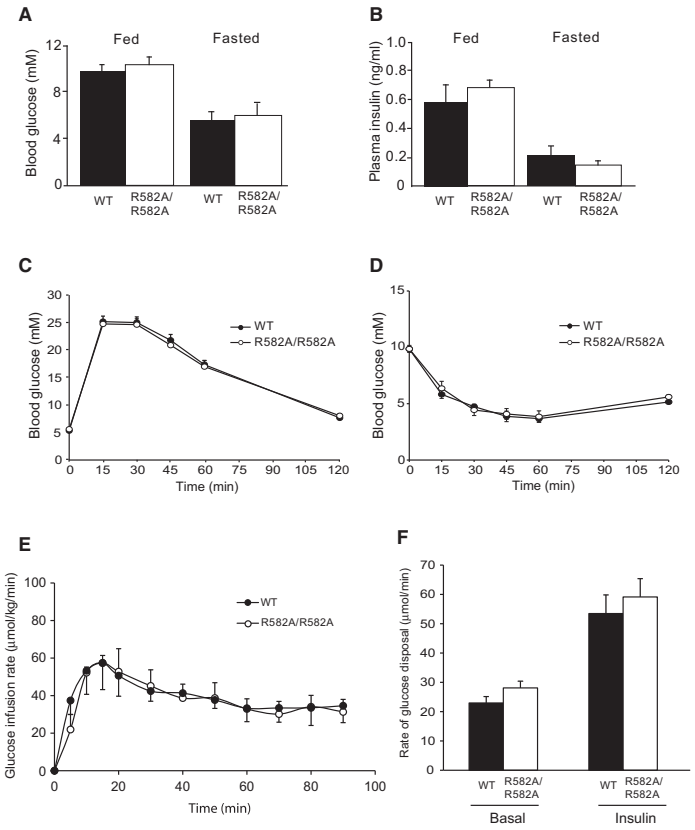

Figure 6. Glucose Tolerance, Insulin Sensitivity, and Plasma Insulin Levels in $GS^{R582A/R582A}$ Knockin and Wild-Type Mice

(A and B) Approximately equal numbers of 2- to 3-month-old male and female mice from indicated genotypes (n = 6–7 animals) were fasted overnight (Fasted) or fed ad libitum (Fed), and blood glucose and plasma insulin levels were determined.

(C and D) Blood glucose concentration in 2- to 3-month-old male $GS^{R582A/R582A}$ knockin and wild-type mice was measured at the indicated times following intraperitoneal administration of 2 mg/g glucose following overnight fast or 0.75 mU/g intraperitoneal insulin injection following 4 hr food removal (n = 7–9 animals/genotype).

(E and F) A euglycaemic-hyperinsulinaemic clamp study was performed as described in the Supplemental Experimental Procedures using four male mice from each genotype (~4 months old). The clamp study started with a bolus of insulin (100 mU/kg), followed by continuous insulin infusion (3.5 mU/kg/min). A variable amount of 12.5% glucose solution was infused to maintain euglycaemia (~5 mmol/l) (E and Figure S3) during a 90 min clamp, and whole-body glucose disposal prior to and at the end of insulin infusion was calculated (F) (n = 4 animals/genotype). Steady-state-specific activities for ^{14}C-glucose during both the basal and hyperinsulinaemic period were achieved (data not shown).

synthesis pathway. In contrast, we (Figure 5D) and others (Reynolds et al., 2005) observed that UDP-glucose levels were reduced in muscles from wild-type animals with insulin, most likely because a sufficient increase in G6P as well as insulin-mediated dephosphorylation would activate GS, thereby elevating the rate of UDP-glucose utilization. Our finding that there was no decrease in UDP-glucose levels with insulin in the muscles of $GS^{R582A/R582A}$ mice correlates with the markedly reduced activity of muscle GS in these animals. Of note, a previous work reported that the overexpression of UDP-glucose pyrophosphorylase and an associated increase in the levels of UDP-glucose do not affect glycogen synthesis in resting and insulin-stimulated mouse skeletal muscles (Reynolds et al., 2005). Finally, although it was not significant, there was a trend for a reduction in GS phosphorylation (Ser641) in muscles from the triple knockin mice in response to insulin (Figure S2E), and we cannot completely rule out the possibility that insulin caused GSK3-independent activation of GS (e.g., PP1-dependent) as a compensatory mechanism.

We observed some dissociation between the reduction of glycogen synthesis (~70%–80%) measured in resting and insulin-stimulated muscles ex vivo (Figures 4A and 4B) and steady-state muscle glycogen content (~50%) in overnight fasted or randomly fed $GS^{R582A/R582A}$ mice compared to those of wild-type animals (Figure 4C). There are several possible explanations. First, it is possible that insulin-independent activation of GS contributes to glycogen synthesis and maintains glycogen levels in skeletal muscle in vivo. It is well known that muscle contraction robustly stimulates GS in order to rapidly restore glycogen content following its breakdown by the action of phosphorylase for energy supply. This occurs via dephosphorylation of GS, but in a PKB/GSK3-independent (McManus et al., 2005) and PP1-dependent manner (Aschenbach et al., 2001). Second, a reduced capacity to synthesize muscle glycogen in GS knockin mice may have led to metabolic adaptations resulting in the muscles of these animals being more efficient at utilizing extracellular glucose and/or fatty acids as substrates for energy production during physical activity, producing a glycogen sparing effect (Pederson et al., 2005). Interestingly, we observed a shift of glucose utilization toward glycolysis in the muscles of GS knockin animals (Figures 5E and 5F). It would be of interest to investigate if oxidation of glucose and fatty acids is increased in muscles of $GS^{R582A/R582A}$ mice during exercise. Third, given that steady-state levels of glycogen are determined by the balance between synthesis and degradation in vivo, it is possible that glycogenolysis was inhibited as a result of some compensatory mechanism in muscles of $GS^{R582A/R582A}$ knockin mice to avoid a profound

depletion of glycogen. We showed that the activity of glycogen phosphorylase in muscle lysate was similar between the GS knockin and wild-type animals (Figure 4E). However, like GS, muscle phosphorylase activity is coordinately regulated by phosphorylation and allosteric stimulators (e.g., AMP) and inhibitors (e.g., ATP, G6P) (Barford et al., 1991), and the cell-free assay is largely a measure of the phospho-dependent activity. Therefore, we cannot rule out the possibility that elevated levels of G6P allosterically inhibited muscle phosphorylase in vivo. It is unlikely that cellular levels of AMP and ATP were altered in resting muscle of GS knockin mice, as activity of the AMP-activated protein kinase, which is regulated by changes in AMP:ATP ratio (Hardie and Sakamoto, 2006), was unchanged (Figures S2C and S2D). To investigate the inhibitory effect of G6P on muscle phosphorylase and glycogen metabolism in vivo, it will be necessary to generate G6P-insensitive phosphorylase knockin animals.

Glycogen synthesis is considered to be of major importance for glucose homeostasis, as muscle with an impaired ability to synthesize glycogen is proposed to attenuate its ability to remove glucose from the circulation, thereby causing insulin resistance and type 2 diabetes. This is based on the assumption that impaired glycogenic activity would be expected to promote accumulation of intracellular G6P levels, which led to the inhibition of hexokinase, thereby reducing the rate of glucose transport (Shulman, 2000). Consistent with this notion, we observed that G6P levels are significantly elevated in muscle from GS knockin compared to that from wild-type mice (Figure 5C). We showed that resting as well as insulin-stimulated glucose transport, assessed by [^{3}H]-2-deoxyglucose uptake, was comparable in isolated soleus and EDL muscles from GS knockin relative to those of wild-type animals (Figure 5A). Although this confirmed that there was no impairment in glucose transport activity in the GS knockin muscles, whether elevated levels of G6P would affect glucose transport under more physiological conditions (e.g., 5–8 mM glucose) could not be addressed under these experimental conditions (no glucose and 1 mM 2-deoxyglucose in the transport buffer). Although 2-deoxyglucose uptake accurately reflects glucose transport activity (Hansen et al., 1994), its intracellular accumulation does not promote feedback inhibition of muscle hexokinase. A previous study showed that in cell-free assays, G6P inhibits hexokinase by 80% at a concentration of 0.5 mM in muscle homogenates, whereas even at a concentration of 30 mM, 2-deoxyglucose-6-phosphate fails to inhibit the enzyme by 80% (Hansen et al., 1994). However, it should be noted that when isolated soleus was incubated under euglycaemic conditions (5.5 mM glucose) ex vivo, glucose utilization, as judged by the sum of glycogen synthesis and glycolysis, was not reduced in knockin muscles (Figure 5E), suggesting that glucose transport was not significantly inhibited. Whether sustained accumulation of G6P occurs under physiological conditions (e.g., following glucose ingestion) in vivo would be interesting, and kinetic analysis of muscle G6P using noninvasive nuclear magnetic resonance analysis can potentially shed light on this. Nonetheless, our GS$^{R582A/R582A}$ knockin mouse model provides a unique opportunity to test if reduced glycogenic activity impairs whole-body glucose homeostasis. We demonstrate that a drastically reduced rate of muscle glycogen synthesis does not necessarily cause impaired whole-body glucose disposal. This is at least in part consistent with a study employing muscle GS-deficient mice that lack glycogen in skeletal and cardiac muscles (Pederson et al., 2005). These mice showed normal blood glucose levels in fasted and fed states and of note they disposed of glucose more effectively than wild-type animals. The mechanism by which GS null mice showed improved clearance of blood glucose was not clear, although the authors speculated that it might be due to sustained elevation of serum insulin in the GS-deficient animals during glucose tolerance tests. The GS null mice were leaner and partially protected against the insulin resistance induced by high-fat-diet feeding. This could be explained by the fact that GS knockout mice had a higher proportion of oxidative fibers with increased phosphorylation of AMPK and ACC, which indicated enhanced capacity in the muscle of GS-deficient mice for more efficient fatty acid and glucose oxidation. As described above, AMPK activity and ACC phosphorylation were not altered in resting muscles between wild-type and GS knockin mice (Figures S2C and S2D). Of note, whole-body insulin sensitivity of GS null mice, judged by glucose infusion rate during a euglycaemic-hyperinsulinaemic clamp, was no different from wild-type animals. Insulin-induced glucose uptake into peripheral tissues was decreased, whereas liver glycogen accumulation was enhanced in the GS knockout mice, indicating muscle insulin resistance. Results from the above study indicate that ablation of muscle GS and a lack of glycogen produce complex metabolic adaptations in various organs to maintain glucose homeostasis. Moreover, the data need to be cautiously interpreted, as the GS deficiency resulted in a cardiac developmental problem, and only ~10% of the GS null mice survive birth (Pederson et al., 2004). Because ~90% of muscle GS knockout mice die shortly after birth, one cannot rule out the possibility that the subset of animals studied was adapted to dispose glucose normally because of the presence of some factor that enabled their survival (Pederson et al., 2005).

Since a reduced capacity to synthesize muscle glycogen did not impair glucose disposal, one might question the importance of muscle glycogen accumulation for blood glucose homeostasis at least in the mouse and also wonder whether mice are an appropriate model to study human metabolism. When expressed as a percentage of total body glycogen, glycogen content in mouse skeletal muscle is only 10% of that in human muscle (Kasuga et al., 2003). In addition, humans have 3- to 8-fold more muscle glycogen than that in liver, whereas fed mice contain five to ten times more total glycogen in liver than in skeletal muscle (Irimia et al., 2010). Therefore, the relative contribution of muscle and hepatic glycogen in controlling glucose homeostasis might be different between mice and humans with hepatic energy reserves of greater importance in mice. Interestingly, some recent reports describing the clinical and metabolic phenotypes of human subjects with mutations/polymorphisms in genes involved in glycogen metabolism suggest a critical role for muscle glycogen accumulation in energy production during exercise, but not for glucose homeostasis. Kollberg et al. have reported that human subjects carrying a homozygous stop mutation in exon 11 in the *GYS1* gene, which is predicted to cause truncation of GS at Arg462, resulted in a loss of GS proteins in both skeletal and heart muscles (Kollberg et al., 2007). Muscle GS-deficient subjects exhibit reduced

exercise capacity with rapid muscle fatigability most likely due to an inability to utilize glycogen as an energy source for ATP generation during muscle contraction. However, they display a normal blood glucose profile when challenged with an oral glucose load. In addition, muscle glycogen depletion due to inactivation of glycogenin-1, caused by biallelic mutations in *GYG1*, resulted in muscle weakness and cardiac arrhythmia; however, the patient exhibited normal glucose tolerance, and levels of fasting glucose and glycated haemoglobin were also normal (Moslemi et al., 2010). Taking our current results and previous findings together, it can be argued that impaired glycogen synthesis as seen in type 2 diabetics may not be a primary cause of insulin resistance; rather it is a consequence due to multiple defects in the insulin signaling pathway, resulting in impaired glucose transport (Shulman, 2000). A reduced rate of glucose transport and concomitant decrease in intramuscular accumulation of G6P would attenuate glycogen synthesis via reduced allosteric activation of GS. Therefore, glucose transport is the critical step for insulin-stimulated glycogen synthesis, and suggests that muscle glucose transport represents a more important therapeutic target for insulin resistance and type 2 diabetes.

We propose that increased levels of intramuscular G6P via glucose transport in response to insulin allosterically stimulate GS, which drives it into glycogen as a feed-forward mechanism, and this effect could be further enhanced when GS is dephosphorylated by inactivation of GSK3 (Patel et al., 2008), as this makes GS more sensitive to G6P. This proposition is consistent with the metabolic control theory proposed by Shulman and Rothman (Shulman and Rothman, 1996). The interplay between allosteric regulation and covalent phosphorylation allows GS to detect small changes in the levels of G6P when elevated upon insulin stimulation. This sophisticated enzyme control system facilitates a sustained influx of glucose, which would prevent a buildup of the levels of intermediates. There are many examples of metabolic enzymes that are coordinately regulated via an interplay between allostery and phosphorylation, and elucidating such interplay would be fundamental in understanding the in vivo regulation of metabolic flux control.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Transfection, and Purification of GS

HEK293 cells were cultured using standard protocols and transfected with 20 μl of polyethylenimine (1 mg/ml) and 5–10 μg of plasmid DNAs per 10 cm dish. Thirty-six hours following transfection, cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer. Lysates were clarified by centrifugation and the supernatant assayed for protein concentration using Bradford reagent. Cell extracts (~70–100 mg protein) generated from cotransfection of untagged GS (wild-type or mutants) and GST-tagged glycogenin were incubated with glutathione Sepharose for 1 hr at 4°C and the resin washed twice with lysis buffer containing 0.5 M NaCl and twice with buffer A (50 mM Tris-HCl [pH 7.5], 0.1 mM EGTA). GST-glycogenin:GS complexes were eluted with 20 mM reduced glutathione. The eluted proteins and BSA standards were resolved on SDS-PAGE and stained with Coomassie blue R250. The band corresponding to GS was scanned and the protein concentration estimated by densitometric analysis using a LI-COR Odyssey imaging system.

Glycogen Synthase Activity

GS activity was measured by a modification of the method of Thomas et al. (Thomas et al., 1968) and described in detail (Lai et al., 2007). Muscles were freeze dried for 3 hr, and 2–3 mg tissue (dry weight) was homogenized (1:400; dry tissue weight:volume) with a rotor-stator homogenizer (Polytron, Kinematica AG) in ice-cold buffer containing 50 mM Tris-HCl (pH 7.8), 100 mM NaF, 10 mM EDTA. Homogenates were centrifuged for 5 min at 3600 × g at 4°C and GS activity measured in supernatants as follows: 20 μl of supernatant was added to 40 μl assay buffer (25 mM Tris-HCl [pH 7.8], 50 mM NaF, 5 mM EDTA, 10 mg/ml glycogen, 1.5 mM UDP-glucose, and 0.5 μCi/ml D-[^{14}C]-UDP-glucose) with 0, 0.3, and 12 mM G6P. Reactions were incubated for 8 min at 37°C and stopped by spotting 50 μl onto squares of filter paper (Whatman ET-31) which were immediately immersed in ice-cold 66% ethanol and washed twice more in 66% ethanol. Dried filters were subjected to scintillation counting.

Determination of Muscle Glucose Utilization

Muscle glycolytic rates were determined by the detritiation of [5-^{3}H]-glucose during the reactions catalyzed by triose phosphate isomerase and enolase. The glucose tracer was dried using a Savant Speedvac concentrator to remove [^{3}H] H_2O accumulated during storage as a consequence of radiolysis. Isolated soleus muscles were incubated in 2 ml Krebs-Ringer bicarbonate (KRB) containing 5.5 mM glucose and 0.5 mCi.mmol [5-^{3}H]-glucose for 40 min at 37°C, gassed continuously with 95%/5% O_2/CO_2. Muscles were snap-frozen in liquid nitrogen and processed for determination of [5-^{3}H] glucose incorporation into glycogen as described for [^{14}C(U)]glucose. [^{3}H] H_2O was isolated by borate complex ion exchange chromatography. Briefly, aliquots (0.5 ml) of conditioned KRB were applied to 1 ml columns of Dowex-1-borate and washed with 2 ml water to elute [^{3}H] H_2O. Glucose forms a borate complex and is retained by the resin. Columns were regenerated with 0.5 M potassium tetraborate. [^{3}H] H_2O was determined by scintillation counting and corrected for leakage of [5-^{3}H]-glucose (typically less than 0.03%).

Statistical Analyses

Data are expressed as mean ± SEM. Statistical analysis was performed by two-tailed Student's t test or one-way ANOVA with Newman-Keuls multiple comparison post hoc test. Differences between groups were considered as statistically significant when $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article at doi:10.1016/j.cmet.2010.10.006.

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Characterization of a canine model of glycogen storage disease type IIIa

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Characterization of a canine model of glycogen storage disease type IIIa

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SUMMARY

Glycogen storage disease type IIIa (GSD IIIa) is an autosomal recessive disease caused by deficiency of glycogen debranching enzyme (GDE) in liver and muscle. The disorder is clinically heterogeneous and progressive, and there is no effective treatment. Previously, a naturally occurring dog model for this condition was identified in curly-coated retrievers (CCR). The affected dogs carry a frame-shift mutation in the GDE gene and have no detectable GDE activity in liver and muscle. We characterized in detail the disease expression and progression in eight dogs from age 2 to 16 months. Monthly blood biochemistry revealed elevated and gradually increasing serum alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) activities; serum creatine phosphokinase (CPK) activity exceeded normal range after 12 months. Analysis of tissue biopsy specimens at 4, 12 and 16 months revealed abnormally high glycogen contents in liver and muscle of all dogs. Fasting liver glycogen content increased from 4 months to 12 months, but dropped at 16 months possibly caused by extended fibrosis; muscle glycogen content continually increased with age. Light microscopy revealed significant glycogen accumulation in hepatocytes at all ages. Liver histology showed progressive, age-related fibrosis. In muscle, scattered cytoplasmic glycogen deposits were present in most cells at 4 months, but large, lake-like accumulation developed by 12 and 16 months. Disruption of the contractile apparatus and fraying of myofibrils was observed in muscle at 12 and 16 months by electron microscopy. In conclusion, the CCR dogs are an accurate model of GSD IIIa that will improve our understanding of the disease progression and allow opportunities to investigate treatment interventions.

INTRODUCTION

Mutations in glycogen debranching enzyme (GDE) gene cause glycogen storage disease type III (GSD III), resulting in accumulation of cytoplasmic glycogen in liver and muscle, the two major tissues for glycogen metabolism (Illingworth and Cori, 1952; Illingworth et al., 1956). GDE is a bifunctional protein having two distinct enzymatic activities: 1,4-α-D-glucan:1,4 α-D-glucan 4-α-D-glycosyltransferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33) (Taylor et al., 1975; Nakayama et al., 2001). Together with glycogen phosphorylase, GDE is responsible for complete degradation of cytoplasmic glycogen. More than 80% of GSD III patients have debranching enzyme deficiencies in both liver and muscle (type IIIa), and most of the rest manifest only liver involvement (type IIIb) (Van Hoof and Hers, 1967; Kishnani et al., 2010).

General clinical manifestations of GSD IIIa include hepatomegaly, fasting hypoglycemia, hyperlipidemia, growth retardation, and variable myopathy and cardiomyopathy. However, disease phenotypes vary widely in patients, most probably caused by different GDE mutations specific to individual families on different genetic and environmental backgrounds (Hobson-Webb et al., 2010; Kishnani et al., 2010). Liver symptoms often appear in childhood and typically improve after puberty, but liver cirrhosis and hepatic adenoma or hepatocellular carcinoma have been reported in some cases (Haagsma et al., 1997; Labrune et al., 1997; Siciliano et al., 2000; Cosme et al., 2005; Demo et al., 2007). Progressive myopathy is the major cause of morbidity in GSD IIIa patients. Muscle weakness is usually not a prominent feature during childhood but can progress with age, rendering some patients wheelchair bound in their third or fourth decade of lives (Momoi et al., 1992; Lucchiari et al., 2007; Kishnani et al., 2010). Periodic acid-Schiff stain (PAS)-positive glycogen storage can be observed in adult patients along with distorted myofiber structures (Kim et al., 2008; Schoser et al., 2008). Glycogen deposition in cardiac muscle has been recognized since 1968 and ventricular hypertrophy is common in GSD IIIa patients (Pearson, 1968; Moses et al., 1989; Lee et al., 1997). Patients with cardiac involvement are at risk of heart failure and life-threatening arrhythmias, yet the actual incidence is relatively low (Miller et al., 1972; Moses et al., 1989; LaBarbera et al., 2010). Consistent with liver and muscle damage, laboratory tests also show elevated serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and creatine phosphokinase (CPK) activities (Coleman et al., 1992; Lee et al., 1995; Lucchiari et al., 2007; Karwowski et al., 2011).

Because the search for an effective treatment for GSD IIIa is ongoing and the pathophysiology of the disease and mechanisms of clinical variability are not well understood, an appropriate animal model that mimics human disease is needed. Recently, GSD IIIa was identified in curly-coated retrievers (CCR) (Gregory et al., 2007). The affected dogs carry a frame-shift mutation predicting deletion of the C-terminal 126 amino acids of GDE and resulting in a GSD IIIa phenotype. Investigation on two affected dogs

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TRANSLATIONAL IMPACT

Clinical issue

Liver and muscle are the major affected tissues in humans with glycogen storage disease type IIIa (GSD IIIa), a hereditary disease caused by mutations in the gene encoding glycogen debranching enzyme (GDE). Liver symptoms often appear in childhood and typically improve after puberty in most individuals, but liver cirrhosis and hepatic adenoma or hepatocellular carcinoma have been reported in some cases. Progressive myopathy and cardiomyopathy are a major cause of morbidity in adults. The natural history of GSD IIIa disease progression has not been well established in human patients and there is no effective treatment. The absence of an adequate animal model is a major obstacle in progressing our understanding of the pathological mechanisms of the disease and evaluating new therapies. Recently, GSD IIIa was identified in curly-coated retrievers (CCRs) that carry a frame-shift mutation predicted to delete the C-terminal 126 amino acids of GDE. The dogs showed no detectable GDE enzyme activity in liver and muscle, but a thorough characterization of these dogs has not been reported.

Results

In this study, the authors characterized in detail the expression and progression of GSD IIIa in affected CCRs. Abnormally high glycogen deposition was found in liver and muscle, and, consistent with liver and muscle damage, high and gradually increasing activity of enzymes including AST, ALT, ALP and CPK were found in serum. In muscle, increased glycogen deposition was accompanied by disruption of the contractile apparatus and fraying of myofibrils. Progressive, age-related liver fibrosis and muscle damage caused by glycogen accumulation were the major features of GSD IIIa in affected dogs.

Implications and future directions

Canine models are emerging as powerful tools to study hereditary diseases and the development of new therapeutic approaches. This work shows that CCRs with GSD IIIa closely resemble human patients, and will be a valuable model for future studies of disease progression, biomarker discovery and treatment interventions. The authors are aiming to evaluate potential treatments such as high-protein diet management, gene therapy, enzyme replacement therapy and blocking glycogen synthesis with existing drugs in this animal model.

showed no detectable GDE enzyme activity in liver and muscle. Analysis of liver biopsies revealed severe glycogen accumulation but there was no evidence of inflammation or fibrosis in the liver. PAS-positive glycogen deposits were observed in a skeletal muscle biopsy taken from one of them at age 14 months (Gregory et al., 2007). However, a thorough characterization of this model and of disease characteristics has not been performed. We have established a breeding colony of the CCR dogs to better understand the phenotype, and to allow for better understanding of disease progression. In this article, we describe this canine GSD IIIa model in extensive biochemical and histological details.

RESULTS

Serum biochemistry

Monthly routine serum biochemistry panels of eight affected dogs revealed gradually increasing liver enzyme activities in these dogs. ALT (normal range 12-118 U/l) increased from 144±36 U/l at 2 months to a peak of more than 700 U/l at 15 months (Fig. 1A). AST (normal range 15-66 U/l) was slightly higher than normal before 6 months (70-80 U/l), then gradually increased to 283±67 U/l at 13 months, followed by a jump to nearly 600 U/l at 14 months (Fig. 1B). The average value of ALP (normal range 5-131 U/l) fluctuated between 200 and 400 U/l, with a trend of increasing with

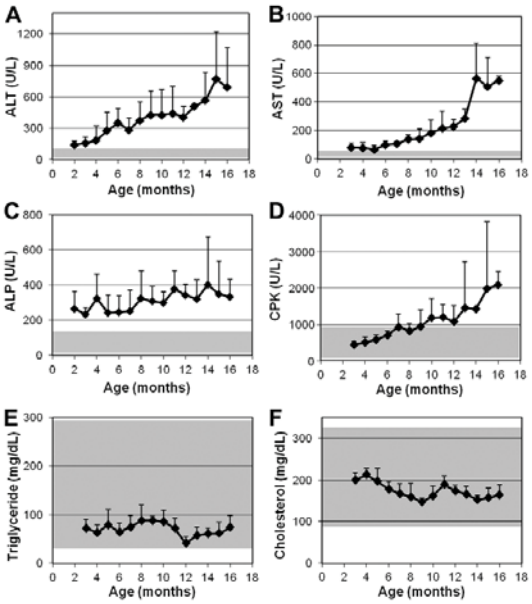

Fig. 1. Serum enzyme activities and lipid levels in GSD IIIa-affected CCRs. (A-F) Blood was collected every month and routine serum biochemistry determinations were performed as indicated. Values show mean + s.d. of 3-6 dogs at each time point. Shaded areas indicate normal ranges.

age (Fig. 1C). The CPK level (normal range 59-895 U/l) in the tested dogs was in the normal range before age 10 months, became slightly above normal from 10 to 12 months, and then continually increased to above 2000 U/l at 16 months (Fig. 1D). Triglycerides (Fig. 1E) and cholesterol (Fig. 1F) concentrations were normal in all dogs throughout the study. All other parameters including glucose, bilirubin, albumin, urea nitrogen, and creatinine were within normal ranges. There was no difference in the growth curves of affected dogs and their normal littermates

Liver and muscle glycogen contents

Liver and muscle biopsies were performed on GSD IIIa dogs after overnight fasting at ages of 4, 12 and 16 months. Liver glycogen content (Fig. 2A) was 209±47 μmol glucose/g tissue at 4 months of age, more than fourfold of that found in a normal dog (~47 μmol glucose/g). The value increased to 269±44 μmol glucose/g at 12 months and dropped to 180±59 μmol glucose/g at 16 months. The decrease in liver glycogen content from age 12 to 16 months was probably due to large-scale replacement of hepatocytes by fibrous tissue, as described later, and correlates well with the observed accelerated increase of serum ALT and AST activities in the same time frame (Fig. 1A,B). Gradually increasing muscle glycogen content was observed in all dogs. As shown in Fig. 2B, muscle glycogen contents were 81±11, 104±12 and 168±49 μmol glucose/g tissue at ages 4, 12 and 16 months, respectively, compared with ~23 μmol glucose/g tissue in a normal dog. The damaging effect of high glycogen content on muscle is evidenced by the sharply

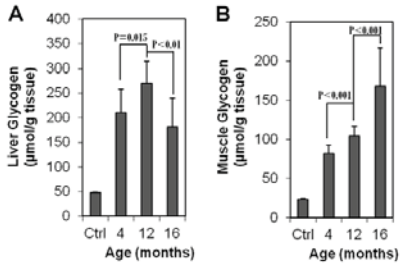

Fig. 2. Fasting glycogen contents in liver and skeletal muscle of GSD IIIa-affected CCRs. (A) Liver glycogen and (B) muscle glycogen at each time point were measured in four dogs at 4 and 12 months, and three dogs at 16 months, with two pieces of the same tissue for each dog. Means + s.d. are shown. Ctrl, average of triplicate specimens from a 6-month-old normal dog.

increased serum CPK and AST activities after 12 months (Fig. 1) and by histopathological analyses described later.

Gross and histological appearance of liver

Liver and muscle are two major tissues affected by glycogen storage. High glycogen turnover rates in liver (Magnusson et al., 1994) predispose this organ to glycogen deposition even at young ages in GSD IIIa. At age 4 months when the first liver biopsy was performed, the livers were enlarged and fragile with relatively smooth surfaces in all dogs (n=4). At 12 months, the livers were severely enlarged and firmer, with small nodules scattered on the surface (n=4). At 16 months, the severely and diffusely enlarged livers were partially or fully involved with large nodules and cirrhosis (n=3). Histological analysis of liver specimens revealed marked glycogen accumulation in hepatocytes at all ages and a gradual disturbance of hepatocellular organization with age. As shown in Fig. 3, hematoxylin and eosin (H&E)-stained paraffin sections (Fig. 3A-C) exhibited the typical vacuolated appearance of glycogen-filled hepatocytes; in high-resolution light microscopy (HRLM) sections stained with PAS-Richardson's stain (Fig. 3D-F), pools of glycogen appear light purple and are well-preserved within hepatocytes. The hepatic architecture appeared normal at 4 months (Fig. 3A,D), mildly altered in local areas at 12 months (Fig. 3B,E) and very distorted at age 16 months, with increased fibrous areas (Fig. 3C,F). A low power view of trichrome-stained paraffin sections illustrates the progressive hepatic fibrosis in canine GSD IIIa (Fig. 4A-C): from periportal fibrosis at 4 months, bridging fibrosis at 12 months and cirrhosis at 16 months. The evolution of these pathological processes was highlighted at higher magnification (Fig. 4D-F).

Progressive muscle damage caused by gradually increased glycogen accumulation

Progressive glycogen accumulation and tissue damage with age was detected in skeletal muscle of GSD IIIa dogs (Fig. 5). At age 4

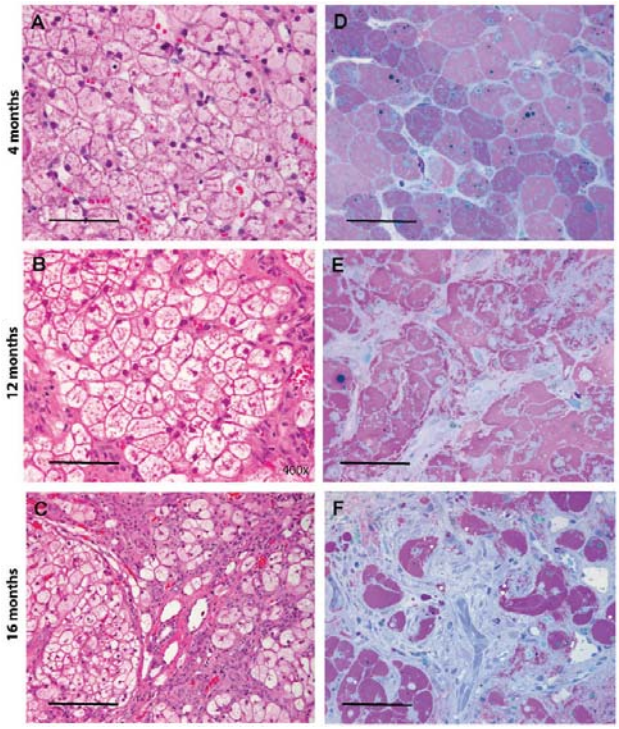

Fig. 3. Marked glycogen accumulation is present in hepatocytes at 4, 12 and 16 months of age. (A-C) Paraffin-embedded, H&E-stained liver sections illustrate the typical vacuolated appearance of glycogen-filled hepatocytes at 4, 12 and 16 months. (D-F) In HRLM sections stained with PAS-Richardson's stain, the glycogen is well preserved and appears light purple. Dense fibrosis is evident in F; fibroblasts stain light blue. Scale bars: 50 μm (A,B,F), 100 μm (C) and 30 μm (D,E).

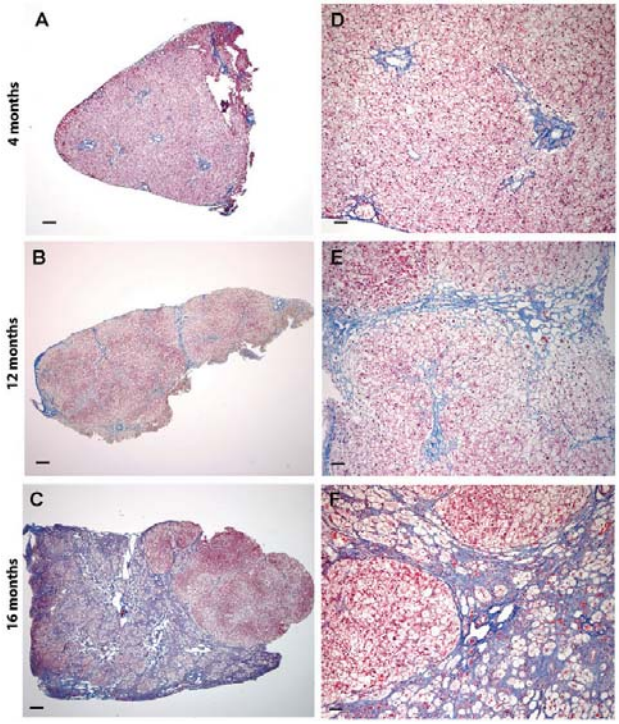

Fig. 4. Progressive hepatic fibrosis is a feature of canine GSD3. (A-C) Progression from periportal fibrosis, to bridging fibrosis to cirrhosis at 4, 12 and 16 months, respectively (paraffin sections, trichrome stain). (D-F) Evolution of these pathological processes at higher magnification (paraffin sections, trichrome stain). Scale bars: 300 μm (A-C) and 100 μm (D-F).

months, only low levels of glycogen accumulated within the cytoplasm of myocytes (Fig. 5A), which is consistent with the observation that symptomatic myopathy is not commonly seen in young patients (Kishnani et al., 2010). All muscle cells were similarly affected. Under electron microscopy, the glycogen granules dispersed among the myofibrils, and small blebs of sarcolemma containing glycogen deposits were readily seen beneath the cytoplasmic membrane (Fig. 5D). At 12 months, the cytoplasmic glycogen began to pool around the periphery of the myotubes. Most cells were involved in glycogen accumulation, with a range from mild to severe involvement (Fig. 5B). The large glycogen pools disrupted the contractile apparatus and caused fraying of myofibrils (Fig. 5E). At 16 months, regions of the sarcoplasm were entirely occupied by cytoplasmic glycogen, displacing the contractile elements (Fig. 5F). The histological findings matched the pattern of gradually increasing glycogen content measured in the muscle tissues (Fig. 2B), and were also in concert with the trend of serum CPK activity (Fig. 1D).

Glycogen deposition in adipocytes in muscle tissues

Fig. 6 demonstrates the glycogen accumulation in adipocytes present in a muscle biopsy from one of the GSD IIIa dogs at 16 months of age. The appearance of PAS-positive substances in adipocytes was an isolated event in our study, but it drew our attention to a potential disturbance of glycogen metabolism in adipocytes in GSD IIIa.

DISCUSSION

GSD III is one of the most common glycogen storage diseases. Currently, disease progression and pathology are not well characterized. Other than symptomatic management, no therapy is available for this condition (Kishnani et al., 2010). There is an urgent need for an animal model to study disease progression and to develop effective therapies that are definitive or targeted and relevant to human treatment modalities. In the past decade, canine models have emerged as a powerful tool for studying hereditary diseases and for the development of new therapeutic approaches. For example, a canine model of GSD I has been established and successfully used for studying disease pathophysiology, long-term complications, and development of gene therapy (Kishnani et al., 2001; Koeberl et al., 2008). The naturally occurring GDE frame-shift mutation in CCR was first identified in 2007 (Gregory et al., 2007). The initial study of two affected dogs confirmed glycogen accumulation in liver and muscle and both dogs showed similar clinical signs to those of the human disease (Gregory et al., 2007). A breeding colony was established to obtain a larger cohort of affected dogs with the aim of understanding pathophysiological disease progression and developing novel therapies. The current study was designed to investigate in detail the natural history of the disease in this canine model.

Hypoglycemia and hyperlipidemia are dominant features in patients with GSD III in infancy and childhood (Hershkovitz et al., 1999; Geberhiwot et al., 2007; Bernier et al., 2008; Kishnani et al.,

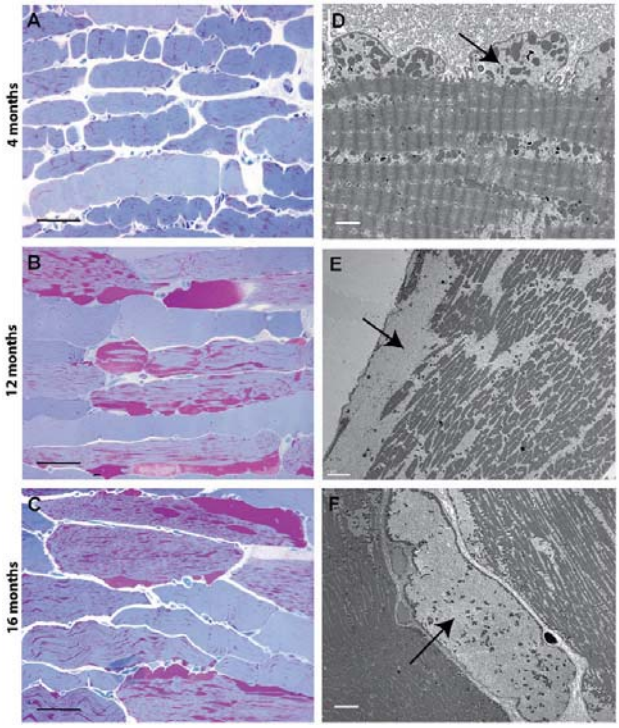

Fig. 5. Progressive cytoplasmic glycogen accumulation occurs in skeletal of GSD3 dogs. (A-C) Progressive accumulation of glycogen in skeletal muscle over time. MetaMorph measurements were 6.5±3.1%, 20.3±7.6% and 17.3±4.7% tissue area occupied by glycogen at 4, 12 and 16 months, respectively (HRLM, PAS-Richardson's stain). (D-F) Ultrastructural changes that occur over time. At 4 months, glycogen accumulates in the cytoplasm and dissects in between myofibrils and just beneath the cytoplasmic membrane, forming small blebs (D). At 12 months, the cytoplasmic glycogen begins to pool and disrupts the contractile apparatus, causing fraying of myofibrils (E). At 16 months, entire regions of cells are filled with glycogen, displacing all contractile elements, leaving only mitochondria to float within the pools of glycogen (F). Arrows indicate glycogen pools. Scale bars: 50 μm (A-C), 2 μm (D), 5 μm (E) and 6 μm (F).

2010). Hyperlipidemia in human patients is possibly caused by increased lipid flux from adipose tissue to the liver as an alternative source of fuel in the setting of hypoglycemia (Bernier et al., 2008). However, none of the eight affected dogs ever displayed signs of hypoglycemia during a 12-hour fasting, which could explain the normal concentrations of triglycerides and cholesterol throughout the study. In addition, there is a great difference in lipoprotein profiles and lipids metabolism between human and dog (Xenoulis and Steiner, 2010).

Liver and muscle damages are common features in GSD IIIa patients, and serum enzyme activities related to these organs are often elevated in the patients. Though abnormalities in serum enzyme activities have been repeatedly reported in previous clinical studies on GSD IIIa (Lucchiari et al., 2007; Karwowski et al., 2011), detailed correlation between enzyme levels and the states of disease progression has not been established. In this study of eight affected dogs, we showed that measurements of both ALT and AST activity were elevated at a young age and continually increased throughout the experiment, indicating progressive liver damage. Though elevations of the two transaminases both indicate liver impairment, ALT is a more direct indicator because it is predominantly found in liver, whereas AST also broadly exists in other tissues, especially muscle (Goessling and Friedman, 2005). The steeper increase of the two enzymes after 12 months of age coincided with the increased liver fibrosis and cirrhosis, as confirmed by histology, though increased AST could have also come from damaged muscle where this enzyme exists in significant amounts (Weibrecht et al., 2010). High levels of ALP were noted in the dogs at different ages. Human ALP exists in several isoforms and various conditions can lead to elevated serum ALP activity, but very high ALP activities are often of liver origin and caused by severe intrahepatic cholestasis or bile duct obstruction (Sapey et al., 2000). Consistently elevated serum ALP activity in the GSD IIIa dogs appears primarily a result of hepatocyte swelling due to cytoplasmic glycogen accumulation, and had little correlation with the extent of fibrosis. CPK catalyzes the conversion of creatine to phosphocreatine, an energy reservoir for the rapid regeneration of ATP through the reverse reaction, in muscle and some neuronal tissues (Wallimann et al., 1992). High serum CPK activity is usually caused by injury or stress to the muscle or heart tissue (Arts et al., 2007). The accelerated increase of CPK after 12 months, correlating with increased muscle glycogen content and damage, implies the disruption of myocyte integrity that mimics the onset of phenotypic myopathy in adult patients. These parameters are useful in clinical diagnosis and evaluation of disease status, but whether they can be used as disease biomarkers needs to be further evaluated in this animal model.

High levels of glycogen were detected in liver at 4 months of age in all dogs. Liver glycogen content increased at 12 months, followed by a significant decrease at 16 months. Progressive liver fibrosis was observed in this dog model. Liver fibrosis was minor at 4 months of age but extensive micronodular and macronodular cirrhosis was found in all three dogs analyzed at 16 months. The

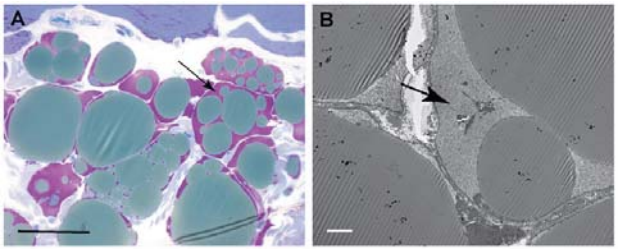

Fig. 6. Accumulation of cytoplasmic glycogen within adipocytes is apparent in 16 month biopsies. (A) Glycogen stains purple at the periphery of fat globules within adipocytes present in skeletal muscle biopsies (HRLM, PAS-Richardson's stain). (B) Electron microscopy demonstrates the finely granular ultrastructure of the glycogen surrounding fat globules in adipocytes. Arrows indicate glycogen pools. Scale bars: 30 μm (A) and 5 μm (B).

decline in glycogen content in liver from age 12 to 16 months was probably due to large-scale replacement of hepatocytes by fibrous tissue, and correlated well with the observed accelerated increase of serum ALT and AST activities. Muscle glycogen content gradually increased with age. Disruption of the contractile apparatus and fraying of myofibrils were directly observed at 12 and 16 months as a result of large cytoplasmic glycogen deposition and correlated with sharply increased serum CPK and AST activities.

In addition to skeletal muscle, varied cardiac muscle involvement has been reported in patients with GSD IIIa. Ventricular hypertrophy is a frequent finding, but overt cardiac dysfunction or symptomatic cardiomyopathy is rare (Moses et al., 1989; Labrune et al., 1991; Hobson-Webb et al., 2010). A recent study demonstrated that a high-protein diet dramatically decreased the left ventricular mass index and serum creatine kinase levels and reversed cardiomyopathy in a patient with GSD IIIa (Dagli et al., 2009), indicating that this treatment is a beneficial therapeutic choice for GSD IIIa patients with cardiomyopathy. Because it is not practical to perform frequent myocardial biopsies on the dogs, cardiac muscle involvement was not a focus in this study. However, we did perform a less invasive electrophysiology study to test the electrical conduction system of the heart in four affected dogs at age 7-8 months, using a single catheter situated within the heart through femoral vein. Of the four dogs studied, two had atrial fibrillation upon electrical stimulation but all other conduction system characteristics were normal. The other two dogs were both within normal limits. Thus, at this time, there is no inclusive conclusion of cardiac involvement in this model.

It is interesting to find significant glycogen accumulation in adipocytes in an affected dog. Adipose tissue is a primary site for lipid storage. Glycogen is also found in adipocytes at a much lower concentrations than in liver and muscle, though its exact role remains unclear (Jurczak et al., 2007; Markan et al., 2010). Early studies suggested that glycogen is converted into fat in the adipose tissue and the dynamic regulation of adipose glycogen may serve as an energy sensor in coordinating glucose and lipid metabolism during the fasted to fed transition (Markan et al., 2010). In normal and nutritionally balanced states, glycogen is histologically invisible in adipose tissue in rats (Fawcett, 1948). However, when rats are fed on a carbohydrate-rich diet after prolonged starvation, adipose glycogen is markedly increased (Tuerkischer and Wertheimer, 1942). In addition, administration of insulin in rats or dogs can result in a transient glycogen accumulation in the adipocytes (Fawcett, 1948). The ability to accumulate glycogen suggests that the synthesis of glycogen in adipocytes is also a dynamic process in GSD IIIa dogs. The clearance of adipocyte glycogen seems to be impaired by the lack of GDE activity in the affected dogs, suggesting that glycogen catabolism takes place through a mechanism similar to that in muscle and liver. To our knowledge, glycogen accumulation in adipose tissues has not been reported in patients with GSD IIIa and other GSDs, though one of us (P.S.K.) has palpated a lipoma-like structure in a GSD III individual. The existence of significant glycogen accumulation in adipocytes is unusual; whether the involvement of adipose tissue in GSD IIIa dogs is a species-specific event or a common feature needs to be further studied.

In this study, we have demonstrated that the naturally occurring GSD IIIa dog model in CCR has a phenotype that closely resembles the human disease, with glycogen accumulation in liver and skeletal muscle that leads to progressive hepatic fibrosis and myopathy. This disease model will help us better understand GSD IIIa disease progression, identify new biomarkers for the disease and develop effective therapies such as enzyme replacement therapy, gene therapy and substrate reduction therapy.

METHODS

Animals

The CCR breeding colony was maintained at Michigan State University. Housing. Mating, rearing of offspring and transport of dogs were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at MSU. Diagnosis of GSD IIIa was confirmed by mutation analysis (Gregory et al., 2007). A total of eight affected dogs of ages ranging from 2 to 6 months were transported to Duke University Medical Center for this study. The animals were reared on a normal canine diet at the Division of Laboratory Animal Resources (DLAR) of Duke University. All animal experiments were approved by the Institutional Animal Care and Use Committee at Duke University and were in accordance with the National Institutes of Health guidelines. Throughout the study, all dogs were on a regular diet that contained approximately 25-30% protein and 45% carbohydrate. For the first year of life, the dogs were fed with Science Diet puppy large breed dry food (Hill's Pet Nutrition, Topeka, KS) by ad-libitum feeding for a few months and then got 2.25 cups twice a day (4.5 cups/day) along with one can of puppy canned food (Science Diet Puppy Gourmet Chicken Entrée) per day. After 1 year, all dogs were switched to normal adult food (Purina Lab Canine Diet 5006; Purina Mills, St Louis, MO). Each dog was given 3 cups of the dry food twice a day (6 cups/day) and also one can of Purina ProPlan Chicken and Rice Entrée (Nestlé Purina PetCare Company, St Louis, MO) wet food per day.

Routine laboratory testing and tissue biopsy

Blood (5 ml) was collected from saphenous or jugular veins for each dog every 4 weeks. Samples were sent to a commercial laboratory for a panel of routine biochemical tests, which included ALT, AST, ALP, CPK, glucose, triglycerides, cholesterol, bilirubin, albumin, urea nitrogen, γ-glutamyl transpeptidase and creatinine. Liver biopsies by laparotomy and skeletal muscle (quadriceps) biopsies were performed on each dog at specified ages under general anesthesia. Fresh tissue specimens were immediately frozen on dry ice and stored at –80°C until used for biochemical analysis, or placed in 3% glutaraldhyde or 10% neutral buffered formalin (NBF) for histology. All dogs were fasted, but offered water for 12 hours prior to the surgical procedures.

Tissue glycogen analysis

Tissue glycogen content was assayed enzymatically using a protocol modified from Kikuchi et al. (Kikuchi et al., 1998). Frozen liver or muscle tissues (50-100 mg) were homogenized in ice-cold de-ionized water (20 ml water/g tissue) and sonicated three times for 20 seconds with 30-second intervals between pulses, using a Misonix XL2020 ultrasonicator. Homogenates were clarified by centrifugation at 12,000 ***g*** for 20 minutes at 4°C. Supernatant (20 µl) was mixed with 55 µl of water, boiled for 3 minutes and cooled to room temperature. Amyloglucosidase (#A1602; Sigma) solution (25 µl diluted 1:50 into 0.1 M potassium acetate buffer, pH 5.5) was added and the reaction incubated at 37°C for 90 minutes. Samples were boiled for 3 minutes to stop the reaction and centrifuged at top speed for 3 minutes in a bench-top microcentrifuge. Supernatant (30 µl) was mixed with 1 ml of Infinity Glucose reagent (Thermo Scientific) and left at room temperature for at least 10 minutes. Absorbance at 340 nm was measured using a Shimadzu UV-1700 spectrophotometer. A reaction without amyloglucosidase was used for background correction for each sample. A standard curve was generated using standard glucose solutions in the reaction with Infinity Glucose reagent (0-120 µM final glucose concentration in the reaction).

Histopathology

Fresh tissues were cut into 1-mm cubes and immersion-fixed overnight in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA). The fixed tissues were then processed into Epon resin for HRLM (Lynch et al., 2005). Tissue sections were cut at 1 µm and stained with PAS-Richardson's stain for glycogen observation. Additional ultrathin sections (70 nm) were cut, stained and examined by electron microscopy. In addition, small pieces of fresh tissue were fixed in 10% NBF and processed into paraffin blocks, cut into 5 µm sections and stained with H&E or trichrome stains.

Statistical analysis of glycogen content

The significance of differences between two different time points was assessed using two-tailed, unequal variance Student's *t*-test; $P<0.05$ was considered to be statistically significant.

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COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

H.Y. and B.S. performed the major experiments and wrote the manuscript; B.L.T. performed histology of tissue biopsies; S.C. performed surgical procedures; J.F. maintained and genotyped the CCR colony; P.S.K., D.D.K. and S.A. provided clinical advice and technical support. B.S. and P.S.K. designed the experiments and supervised the study.

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Molecular Mechanism by Which AMP-Activated Protein Kinase Activation Promotes Glycogen Accumulation in Muscle

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OBJECTIVE—During energy stress, AMP-activated protein kinase (AMPK) promotes glucose transport and glycolysis for ATP production, while it is thought to inhibit anabolic glycogen synthesis by suppressing the activity of glycogen synthase (GS) to maintain the energy balance in muscle. Paradoxically, chronic activation of AMPK causes an increase in glycogen accumulation in skeletal and cardiac muscles, which in some cases is associated with cardiac dysfunction. The aim of this study was to elucidate the molecular mechanism by which AMPK activation promotes muscle glycogen accumulation.

RESEARCH DESIGN AND METHODS—We recently generated knock-in mice in which wild-type muscle GS was replaced by a mutant (Arg582Ala) that could not be activated by glucose-6-phosphate (G6P), but possessed full catalytic activity and could still be activated normally by dephosphorylation. Muscles from GS knock-in or transgenic mice overexpressing a kinase dead (KD) AMPK were incubated with glucose tracers and the AMPK-activating compound 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) ex vivo. GS activity and glucose uptake and utilization (glycolysis and glycogen synthesis) were assessed.

RESULTS—Even though AICAR caused a modest inactivation of GS, it stimulated muscle glycogen synthesis that was accompanied by increases in glucose transport and intracellular [G6P]. These effects of AICAR required the catalytic activity of AMPK. Strikingly, AICAR-induced glycogen synthesis was completely abolished in G6P-insensitive GS knock-in mice, although AICAR-stimulated AMPK activation, glucose transport, and total glucose utilization were normal.

CONCLUSIONS—We provide genetic evidence that AMPK activation promotes muscle glycogen accumulation by allosteric activation of GS through an increase in glucose uptake and subsequent rise in cellular [G6P]. ***Diabetes*** **60:766–774, 2011**

AMPK is a major regulator of cellular and whole-body energy homeostasis that coordinates metabolic pathways to balance nutrient supply with energy demand (1–4). In response to cellular stress, AMPK inhibits anabolic pathways and stimulates catabolic pathways to restore cellular energy charge. In skeletal muscle, AMPK is activated under energy-consuming conditions such as during contraction and also energy-depleting processes such as hypoxia, which leads to an increase in fatty acid oxidation, glucose uptake, and inhibition of protein synthesis (1,5). The most well established function of AMPK activation in muscle is to stimulate glucose transport by promoting the redistribution of GLUT4 from intracellular compartments to the cell surface (5–7).

The resulting increase in glucose transport and phosphorylation of glucose by hexokinase II leads to an increase in the intracellular level of glucose-6-phosphate (G6P) (8,9). G6P can be used for the synthesis of glycogen or metabolized in the glycolytic pathway to generate ATP. During glycogen synthesis, G6P is converted to uridine diphosphate (UDP) glucose, and the glucosyl moiety from UDP glucose is used to elongate a growing glycogen chain through α-1,4-glycosidic bonds by the action of glycogen synthase (GS) (10,11). There are two GS isoforms in mammals encoded by separate genes. *GYS1*, encoding the muscle isoform, is expressed in muscle and many other organs, including kidney, heart, and brain, whereas *GYS2*, encoding the liver GS isoform, is expressed exclusively in the liver (11). GS activity of both isoforms is regulated by G6P, an allosteric activator, and by covalent phosphorylation, which inhibits enzyme activity (10).

Carling and Hardie (12) reported that AMPK phosphorylates muscle GS at site 2 (Ser8 [amino acid numbering starts from the initiator methionine residue] in human, mouse, and rat), a known inhibitory site of the enzyme, in cell-free assays. Recent work has shown in intact skeletal muscle tissue that acute stimulation of AMPK by a pharmacologic activator, 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), promotes phosphorylation of GS at site 2 (13), resulting in a decrease in enzymatic activity (13–15). From these findings, it was anticipated that activation of AMPK would reduce muscle glycogen levels. However, in apparent conflict with this anticipation, long-term/chronic activation of AMPK increases glycogen storage in skeletal (16,17) and cardiac (18) muscles. Some have speculated that AMPK-mediated increases in glucose transport and the subsequent elevation of intracellular [G6P] are able to allosterically stimulate GS and thus glycogen synthesis by overriding the inhibitory phosphorylation of GS in muscles (8,9).

This hypothesis, however, has not been directly tested, mainly because there are currently no experimental or assay systems to assess G6P-mediated regulation of GS in vivo. GS activity is routinely assayed in vitro using cell/tissue extracts in which the rate of incorporation of UDP-[^{14}C]glucose into glycogen is measured in the absence or presence of G6P (19). GS activity in the presence of saturating concentrations of G6P is independent of the state of phosphorylation, and the activity ratio in the absence of

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G6P relative to that in the presence of G6P is used as an index of GS activity. However, it has been virtually impossible to prove that G6P activates GS in vivo or to assess its physiologic significance because G6P binds noncovalently to GS and therefore dissociates from it when muscle tissue is homogenized in a protein extraction buffer.

We have recently identified a key residue, Arg582, which is located in a highly basic segment comprising a putative G6P-binding pocket at the C-terminus of GS (20,21). Substitution of Arg582 to Ala (R582A) caused a complete loss of allosteric activation of GS by G6P without affecting phosphorylation-dependent enzymatic activity and robustly reduced insulin-mediated glycogen synthesis in skeletal muscle (22). To investigate the physiologic involvement of allosteric activation of GS in regulating muscle glycogen metabolism in vivo, a knock-in mouse expressing a G6P-insensitive GS mutant ($GS^{R582A/R582A}$ mouse) has recently been generated (22). Using this mouse model, we demonstrate here that acute activation of AMPK promotes muscle glycogen synthesis through allosteric activation of GS through increasing glucose uptake and the subsequent rise in intracellular [G6P].

RESEARCH DESIGN AND METHODS

Materials. D-[U-^{14}C]glucose-1-phosphate was purchased from Hartmann Analytic GmbH (Brunswick, Germany). All other radiochemicals were from Perkin Elmer (Buckinghamshire, U.K.). Human insulin (Actrapid) was from Novo-Nordisk (Bagsværd, Denmark). AICAR was from Toronto Research Chemicals (Ontario, Canada). Wortmannin was from Tocris (Avonmouth, U.K.). Horseradish peroxidase-conjugated secondary antibodies were from Thermo Fisher (Northumberland, U.K.). All other chemicals, unless specified otherwise, were obtained from Sigma (Poole, U.K.).

Animals. Animal studies were approved by the University of Dundee Ethics Committee and performed under a U.K. Home Office project license. C57BL/6 J mice were obtained from Harlan (Leicestershire, U.K.). Generation of the muscle $GS^{R582A/R582A}$ knock-in (22) and transgenic AMPK kinase dead (K45R) (23) mice has been previously described.

Antibodies. Total acetyl CoA carboxylase (ACC), phospho ACC1 (Ser^{79}, also detects equivalent site on mouse ACC2 at Ser^{212}), total raptor, phospho raptor (Ser^{792}), total glycogen synthase (GS), phospho GS (Ser^{641}), total AMPKα, phospho AMPKα (Thr^{172}), and phospho protein kinase B (PKB; Thr^{308}) antibodies were from Cell Signaling Technology (Beverly, MA). Hexokinase II antibody (C-14) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho GS antibodies (Ser^{8} and $Ser^{8/11}$) (24) and the total AMPKα1 and AMPKα2 antibodies used for immunoprecipitation were generated and donated by Professor D. Grahame Hardie (University of Dundee). GLUT4 antibody was generated and donated by Professor Geoffrey Holman (University of Bath). Generation of the TBC1D1 (total [S279C, 1st bleed] and antiphospho Ser^{231} [S131C, 2nd bleed]) (25), phosphorylase (total [S956A, 2nd bleed] and antiphospho Ser^{15} [S960A, 1st bleed]), and total PKBα (S742B, 2nd bleed) antibodies (26) has been previously described.

Incubation of isolated muscle. Mice were killed by cervical dislocation, and extensor digitorum longus (EDL) muscles were rapidly removed and mounted on an incubation apparatus as described (26). Muscles were incubated in 2 mL Krebs-Ringer bicarbonate (KRB) buffer (in mmol/L: 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 24.6 $NaHCO_3$, pH 7.4) containing 5.5 mmol/L D-glucose for 40 min at 37°C gassed continuously with 95% O_2 and 5% CO_2. At the end of the incubation period muscles were frozen in liquid nitrogen and stored at −80°C.

Measurement of glucose transport. EDL muscles were isolated and 2-deoxy-[^{3}H]glucose uptake was measured as described (27). Briefly, muscles were incubated in 2 mL KRB containing 2 mmol/L pyruvate for 40 min at 37°C. Muscles were transferred to 2 mL KRB containing 1 mmol/L 2-deoxyglucose (1.5 mCi/mmol 2-deoxy-D-[1,2-^{3}H(N)]glucose) and 7 mmol/L D-mannitol (0.064 mCi/mmol D-[1-^{14}C]mannitol) and incubated for an additional 10 min at 30°C. Muscles were frozen in liquid nitrogen and acid hydrolysates subjected to scintillation counting (27).

Measurement of glycogen synthesis. D-[U-^{14}C]glucose incorporation into muscle glycogen was determined as previously described (26). Briefly, EDL muscles were incubated in 2 mL KRB containing 5.5 mmol/L D-glucose and 0.1 mCi/mmol D-[U-^{14}C]glucose for 40 min at 37°C. Muscles were frozen in liquid nitrogen and [^{14}C]glycogen extracted by ethanol precipitation from KOH digests as described (26).

Measurement of glycolysis. The rate of glycolysis was determined by the detritiation of [5-^{3}H]glucose as described (22). Briefly, muscles were incubated in 2 mL KRB containing 5.5 mmol/L glucose and 0.5 mCi/mmol [5-^{3}H]glucose for 40 min at 37°C. Muscles were frozen in liquid nitrogen, and the rate of [5-^{3}H]glucose incorporation into glycogen was determined as described for D-[U-^{14}C]glucose. [^{3}H]H_2O was isolated from conditioned KRB by borate complex ion exchange chromatography and measured by scintillation counting.

Preparation of muscle lysates. Muscle lysates were prepared as described (26). Homogenates were clarified at 3,000*g* for 10 min at 4°C, and protein concentration was estimated using Bradford reagent and bovine serum albumin (BSA) as standard. Lysates were frozen in liquid nitrogen and stored at −80°C.

Immunoblotting. Muscle extracts (20–30 μg) were denatured in SDS sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene fluoride membrane. Membranes were blocked for 1 h in 20 mmol/L Tris-HCl (pH 7.5), 137 mmol/L NaCl, and 0.1% (v/v) Tween-20 (TBST) containing 5% (w/v) skimmed milk. Membranes were incubated in primary antibody prepared in TBST containing 5% (w/v) BSA overnight at 4°C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagent.

Assay of glycogen synthase and phosphorylase. Muscle homogenates (25 μg) were assayed for glycogen synthase and phosphorylase activity (reverse direction) by measuring the incorporation of UDP-[U-^{14}C]glucose and [U-^{14}C] glucose-1-phosphate respectively into glycogen, as described (22). Results are expressed as the activity ratio in the absence and presence of 10 mmol/L G6P (glycogen synthase) or 2 mmol/L AMP (phosphorylase).

AMPK activity assay. AMPK was immunoprecipitated from 30 μg lysate with antibodies against the α1 and α2 subunits and assayed for phosphotransferase activity toward *AMARA* peptide (AMARAASAAALARRR) using [γ-^{32}P]ATP, as previously described (28).

Assay of muscle glycogen. Frozen muscles were digested in 100 μL of 1 mol/L KOH for 20 min at 80°C. The pH was adjusted to 4.8 with 50 μL of 4 mol/L acetic acid and 250 μL of 4 units/mL amyloglucosidase (*Aspergillus niger*) in 0.2 mol/L sodium acetate (pH 4.8 added). Samples were incubated for 2 h at 40°C, clarified at 16,000*g* for 10 min, and neutralized with NaOH. Glucose released from glycogen was determined using a commercial hexokinase/G6P dehydrogenase (G6PDH) coupled assay (Amresco, Solon, OH) using D-glucose as a standard.

Assay of muscle G6P. G6P was assayed fluorometrically in $HClO_4$ extracts of EDL muscle, as previously described (22).

Statistical analyses. Data are expressed as means ± SEM. Statistical analysis was performed by unpaired, two-tailed Student *t* test or one-way or two-way ANOVA with Dunnett post hoc test. Differences between groups were considered statistically significant when $P < 0.05$.

RESULTS

Pharmacologic activation of AMPK leads to inactivation of muscle glycogen synthase. We first measured the effect of the pharmacologic AMPK activator, AICAR, on AMPK activity in EDL muscle (composed mainly of fast-twitch, glycolytic fibers) isolated from male C57BL/6 mice. EDL was used because the effects of AICAR on AMPK activity and glucose uptake are reported to be robust in this muscle (29). As previously reported (27), incubation of EDL ex vivo with AICAR promoted robust phosphorylation of the AMPKα catalytic subunit at Thr172 in the T-loop and activation of both AMPKα1 (~twofold) and AMPKα2 (~fourfold) compared with unstimulated muscle (Fig. 1*A* and *B*). Consistent with these observations, AICAR treatment increased phosphorylation of known AMPK substrates such as ACC2 at Ser212, raptor at Ser792 (30), and TBC1D1 at Ser231 (25,31) (Fig. 1*B*).

We next assessed the effect of AICAR on phosphorylation and activity of GS in isolated EDL muscle. Muscles were incubated in parallel with GS-activating (insulin) and GS-inhibiting (adrenaline) hormones as controls. AICAR treatment resulted in a modest but significant decrease in GS activity (~10%) (Fig. 2*A*). However, in contrast to previous studies (13,14), no detectable change in the phosphorylation of GS at Ser8, an inhibitory site that is

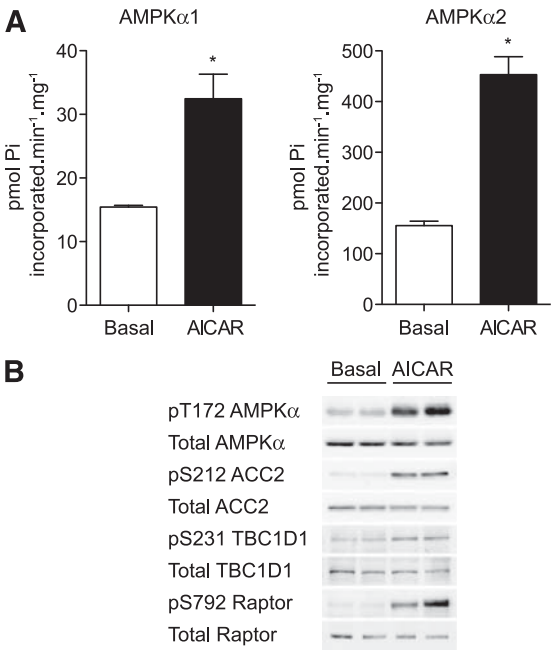

FIG. 1. AICAR robustly stimulates activation of AMPK in EDL muscle. EDL muscles from male C57BL/6J mice (7–10 weeks old) were incubated with vehicle or 2 mmol/L AICAR for 40 min in KRB containing 5.5 mmol/L glucose. *A*: AMPK was immunoprecipitated from muscle lysates using anti-AMPKα1 or anti-AMPKα2 antibodies and assayed for activity using AMARA peptide. Assays were performed in duplicate from muscles derived from four mice and are expressed as mean ± SEM. *B*: Lysates were immunoblotted to assess phosphorylation of components of the AMPK pathway. Results are representative of experiments performed on tissues from at least three mice. *$P < 0.05$.

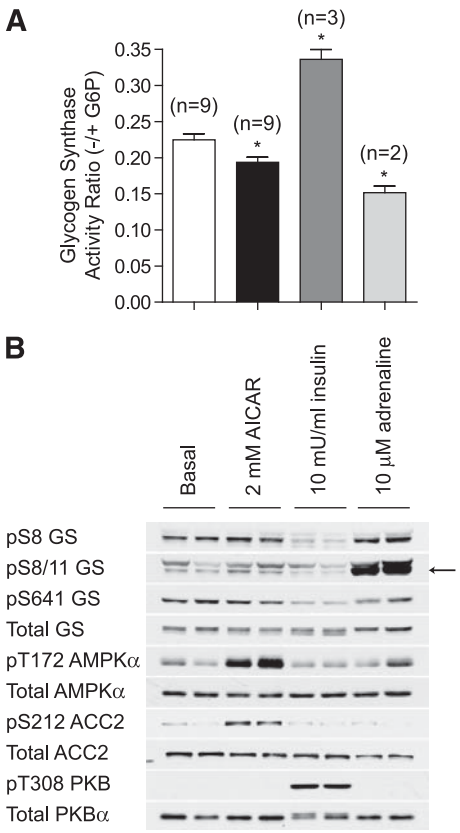

FIG. 2. AICAR modestly inhibits GS activity in vitro. EDL muscles from C57BL/6J mice were incubated with vehicle, 2 mmol/L AICAR, 10 mU/mL insulin, or 10 μmol/L adrenaline for 40 min in KRB containing 5.5 mmol/L glucose. *A*: GS activity was measured in muscle homogenates as described in RESEARCH DESIGN AND METHODS. Results are presented as mean ± SEM from the indicated number (*n*) of mice. *B*: Alternatively, lysates were immunoblotted for GS phosphorylation with the indicated antibodies. *$P < 0.05$ compared with basal.

targeted by AMPK (12), was observed (Fig. 2*B*). Because phosphorylation of Ser8 is known to promote subsequent phosphorylation of Ser11 by casein kinase 1, which cannot be detected by Ser8 phospho-specific antibody, we also monitored dual phosphorylation of Ser8 and Ser11 by a phospho-specific antibody that detects only when both sites are phosphorylated. AICAR failed to induce a detectable change in the phosphorylation of Ser8 and Ser11, whereas adrenaline robustly increased phosphorylation of these sites, correlating with a marked decrease in GS activity (Fig. 2*A* and *B*). AICAR did not modify phosphorylation of other key regulatory sites such as Ser641, whereas insulin promoted dephosphorylation of this site likely via the PKB/GS kinase 3 (GSK3) pathway (Fig. 2*B*), resulting in a ~1.7-fold increase in GS activity (Fig. 2*A* and *B*), as previously reported (32).

AICAR stimulates muscle glycogen synthesis independent of the phosphoinositide-3 kinase pathway. A well-established physiologic role of AMPK in muscle is to stimulate glucose transport (5). Incubation of EDL with AICAR or insulin significantly increased 2-deoxy-[^{3}H]glucose uptake by ~twofold and ~1.5-fold, respectively (Fig. 3*A*). AICAR also caused an increase (2.5-fold) in the levels of G6P compared with resting EDL (Fig. 3*B*). Although chronic/continuous AICAR treatment is known to promote glucose transport and phosphorylation at least partially through an increase in the levels of GLUT4 and hexokinase II- (16,17) short-term incubation (40 min) did not cause detectable changes in the amount of these proteins (Fig. 3*E*). We next measured glycogen synthesis by monitoring incorporation of D-[^{14}C-U]glucose into glycogen in response to AICAR or insulin. Both AICAR and insulin stimulated glycogen synthesis by ~1.6-fold (Fig. 3*C*), which was associated with a significant increase (1.4-fold) in muscle glycogen concentration (Fig. 3*D*).

The effect of AICAR on both glucose uptake and glycogen synthesis was distinct from those by insulin, and the phosphoinositide-3 kinase pathway as wortmannin, a selective phosphoinositide-3 kinase inhibitor, abolished insulin-stimulated glucose transport and glycogen synthesis, but not in response to AICAR (Fig. 4*A* and *B*).

AICAR has no effect on muscle glycogen phosphorylase activity. Net glycogen content and incorporation of [^{14}C]glucose are dependent on both glycogen synthesis and degradation. Consequently, the increased glycogen content observed in AICAR-treated muscles could be partly

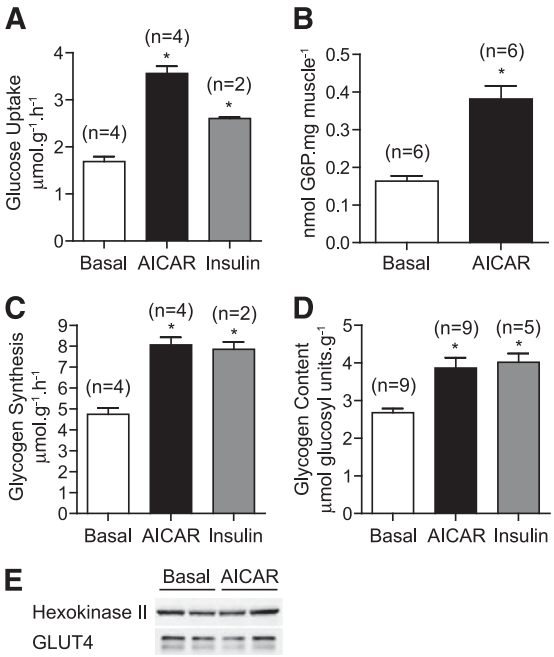

FIG. 3. AICAR stimulates glucose uptake and storage into glycogen. EDL muscles from C57BL/6J mice were incubated with vehicle, 2 mmol/L AICAR, or 10 mU/mL insulin for 40 min in KRB containing 2 mmol/L pyruvate. *A*: Muscles were transferred to vials containing 2-deoxy-[^{3}H] glucose and glucose transport assayed as described in RESEARCH DESIGN AND METHODS. *B*: Alternatively, EDL muscles incubated with vehicle or 2 mmol/L AICAR for 40 min were snap frozen in liquid nitrogen and glucose-6-phosphate (G6P) levels assayed as described in RESEARCH DESIGN AND METHODS. *C*: Isolated EDL muscles were incubated with the indicated stimuli for 40 min in KRB containing D-[U-^{14}C]glucose (5.5 mmol/L), and the rate of glucose incorporation into glycogen was determined as described in RESEARCH DESIGN AND METHODS. *D*: Alternatively, EDL muscles were incubated in KRB containing unlabeled glucose (5.5 mmol/L) for 1 h, and glycogen content was determined by digestion of KOH extracts with amyloglucosidase as described in RESEARCH DESIGN AND METHODS. *E*: EDL muscles were incubated with vehicle or 2 mmol/L AICAR for 40 min and lysates immunoblotted with the indicated antibodies. Results are presented as mean ± SEM from the indicated number (*n*) of mice. *$P < 0.05$ compared with basal.

due to a decrease in glycogen degradation. To rule out this possibility, we assessed the activity of muscle glycogen phosphorylase, a rate-limiting enzyme in glycogen degradation. Phosphorylase activity and phosphorylation at Ser15, an activating residue that is catalyzed by phosphorylase kinase, were both unchanged in response to AICAR or insulin (Fig. 5*A* and *B*). To confirm that our assay was sensitive enough to detect changes in phosphorylase activity, isolated EDL muscles were also incubated in the presence of adrenaline, an activator of phosphorylase through a phosphorylase kinase-dependent pathway. As expected, adrenaline caused a significant activation of phosphorylase (twofold) that was accompanied by an increase (~50%) in phosphorylation of Ser15 (Fig. 5*B*).

Catalytic activity of AMPK is required for AICAR-stimulated muscle glycogen synthesis. To establish that AICAR-stimulated glycogen synthesis is mediated through AMPK and not by off-target action(s) of the compound, we assessed the effect of AICAR on glycogen synthesis in EDL from mice expressing catalytically inactive/kinase dead (KD) AMPKα2 (23). Immunoblot analysis using anti-panAMPKα antibody confirmed that KD AMPK, epitope-tagged with Myc, displayed a slightly slower mobility and endogenous AMPKα was absent (Fig. 6*D*). As previously reported, AMPKα2 activity was largely eliminated, and AMPKα1 was also substantially reduced at rest in EDL from AMPK KD animals (Fig. 6*A*), an effect likely due to the displacement of endogenous α2 and also α1 from βγ heterotrimers by over-expressed KD α2, as has previously been suggested (23). Consistent with previous studies (33), AICAR significantly stimulated both AMPKα1 and AMPKα2 activity in wild-type mice, whereas neither AMPKα1 nor α2 activity was significantly increased in muscles from AMPK KD mice (Fig. 6*A*). AICAR caused a robust increase in ACC2 phosphorylation, which was partially suppressed (~30–40%) in AMPK KD mice (Fig. 6*D*), as previously reported (33).

We next measured the effect of AICAR on GS activity in EDL isolated from AMPK KD or wild-type littermate control animals. In unstimulated muscles, GS activity was significantly higher (about twofold) in AMPK KD compared with wild-type mice (Fig. 6*B*), which was associated with a modest decrease in GS phosphorylation at both Ser8 and Ser641 (Fig. 6*D*). AICAR caused a modest (~10%) decrease in GS activity in wild-type mice but not in AMPK KD animals, demonstrating that AMPK catalytic activity, most likely α2 activity (13), is required for GS inactivation by AICAR (Fig. 6*B*). AICAR did not significantly promote single Ser8 and dual Ser8/11 phosphorylation in EDL from wild-type or AMPK KD mice. Insulin promoted GS dephosphorylation on Ser641 and activation in muscles of wild-type and AMPK KD mice to a relatively similar degree (Fig. 6*B* and *D*). Insulin did not cause a significant change in Ser8 phosphorylation, although some muscle showed a slightly reduced phospho signal on this site (Fig. 6*D*). We next measured glycogen synthesis and observed that AICAR-stimulated increases were abolished in AMPK KD animals, whereas insulin stimulated glycogen synthesis in both genotypes to the same extent (Fig. 6*C*). Total protein expression and phosphorylation of phosphorylase was similar between wild-type and AMPK KD muscles in both resting and AICAR-treated mice (Fig. 6*D*).

Allosteric activation of GS is required for AICAR-stimulated muscle glycogen synthesis. To establish that AICAR-stimulated glycogen synthesis is mediated through allosteric activation of GS by G6P, we have used G6P-insensitive GS knock-in mice in which a critical G6P permissive residue (Arg582) is changed to Ala ($GS^{R582A/R582A}$). We have recently reported that mutant GS derived from $GS^{R582A/R582A}$ mouse skeletal muscle is completely resistant to G6P but retains its capacity to be activated through dephosphorylation by GSK3 inhibition in response to insulin (22).

As reported previously, glycogen synthesis in resting EDL was comparable between wild-type and $GS^{R582A/+}$ mice, and there was a substantial decrease (~70–80%) in glycogen synthesis in $GS^{R582A/R582A}$ animals compared with wild-type or $GS^{R582A/+}$. AICAR stimulated glycogen synthesis in wild-type and $GS^{R582A/+}$ mice by ~twofold and ~1.5-fold, respectively, whereas AICAR had no effect on glycogen synthesis in $GS^{R582A/R582A}$ animals (Fig. 7*A*). There was a trend that AICAR modestly inhibited glycogen synthesis in $GS^{R582A/R582A}$ mice, possibly through deactivation of GS or stimulation of phosphorylase. To rule

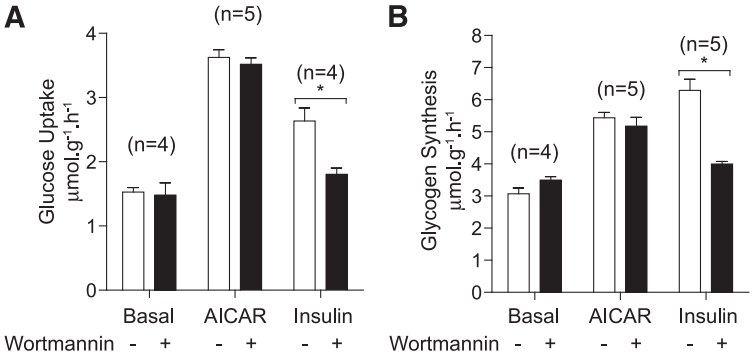

FIG. 4. AICAR-stimulated glucose disposal is independent of phosphoinositide-3 (PI-3) kinase. EDL muscles from C57BL/6J mice were incubated with vehicle (0.2% DMSO) or 100 nmol/L wortmannin for 20 min in KRB/pyruvate. *A*: Muscles were transferred to fresh KRB/glucose containing matched vehicle/compound and the indicated stimuli for 40 min. Finally, glucose transport was assayed using 2-deoxy-[^{3}H]glucose as described in RESEARCH DESIGN AND METHODS. *B*: Alternatively, muscles preincubated with vehicle or wortmannin as described in (*A*) were transferred to KRB containing D-[U-^{14}C]glucose (5.5 mmol/L) and the indicated stimuli and incubated for 40 min. The rate of glucose incorporation into glycogen was determined as described in RESEARCH DESIGN AND METHODS. Results are expressed as mean ± SEM from the indicated number (*n*) of mice. **P* < 0.05 compared with basal.

out the possibility that the reduced glycogen synthesis observed in $GS^{+/R582A}$ and $GS^{R582A/R582A}$ mice was caused by reduced glucose transport or altered AMPK activity, or both, these parameters were measured in isolated EDL muscle in the presence or absence of AICAR. We observed no difference in basal or in AICAR-stimulated glucose uptake across all genotypes (Fig. 7*B*). We also confirmed that resting and AICAR-stimulated AMPK activity (α1 and α2) were similar between wild-type and $GS^{R582A/R582A}$ mice (Fig. 7*C*).

Interestingly, despite the significant impairment in glycogen synthesis, overall glucose utilization, as determined by metabolism of [5-^{3}H]glucose was also similar between wild-type and $GS^{R582A/R582A}$ mice (Fig. 7*D*). The decrease in glycogen synthesis in $GS^{R582A/R582A}$ mice was compensated by a significant increase in the rate of glycolysis in both resting and AICAR-stimulated muscles. Consistent with this finding, the rate of lactate release was also elevated under resting and AICAR-stimulated conditions in $GS^{R582A/R582A}$ mice compared with wild-type (Fig. 7*E*). We confirmed that GS activity in resting EDL muscles was comparable between wild-type and $GS^{R582A/R582A}$ mice when assayed in the absence of G6P, whereas the robust G6P-mediated activation observed in wild-type mice was completely abolished in the muscle of $GS^{R582A/R582A}$ mice (Fig. 7*F*). AICAR modestly deactivated muscle GS in both wild-type and $GS^{R582A/R582A}$ animals (Fig. 7*F*). Potentially, hypophosphorylation of GS by inhibition of GSK3 may compensate for the lack of allosteric activation by G6P in $GS^{R582A/R582A}$ mice. Accordingly, we co-incubated EDL muscle with AICAR and the GSK3 selective inhibitor, CT99021, and observed a significant, albeit modest increase in glycogen synthesis compared with muscle treated with AICAR alone (Supplementary Fig. 1).

DISCUSSION

We (8) and others (16,17) previously reported that AICAR treatment caused an increase in muscle glycogen levels; however, whether this was mediated through AMPK has not been clearly demonstrated. AICAR (Z-riboside) is a widely used AMPK activator that is taken up into cells by adenosine transporters and converted by adenosine kinase to the monophosphorylated derivative ZMP (34). ZMP binds to the γ subunit of AMPK and mimics the effect of AMP on the allosteric activation of the kinase and also on inhibition of dephosphorylation (35). However, because AICAR has been found to produce AMPK-independent effects due to binding of ZMP to other AMP/ZMP-regulated enzymes and also to unknown off-target effects (34), we wished to establish that AICAR-stimulated glycogen synthesis occurs in an AMPK-dependent manner.

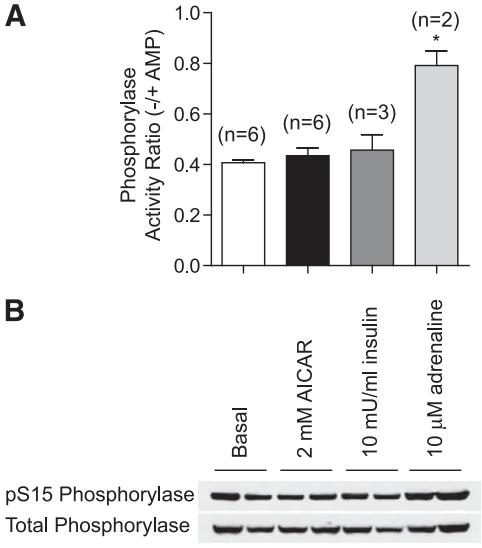

FIG. 5. AICAR has no effect on glycogen phosphorylase activity in vitro. EDL muscles were treated as described in Fig. 2 and lysates were assayed for phosphorylase activity (*A*) or immunoblotted with the indicated antibodies (*B*). Results are presented as mean ± SEM from the indicated number (*n*) of mice. **P* < 0.05 compared with basal.

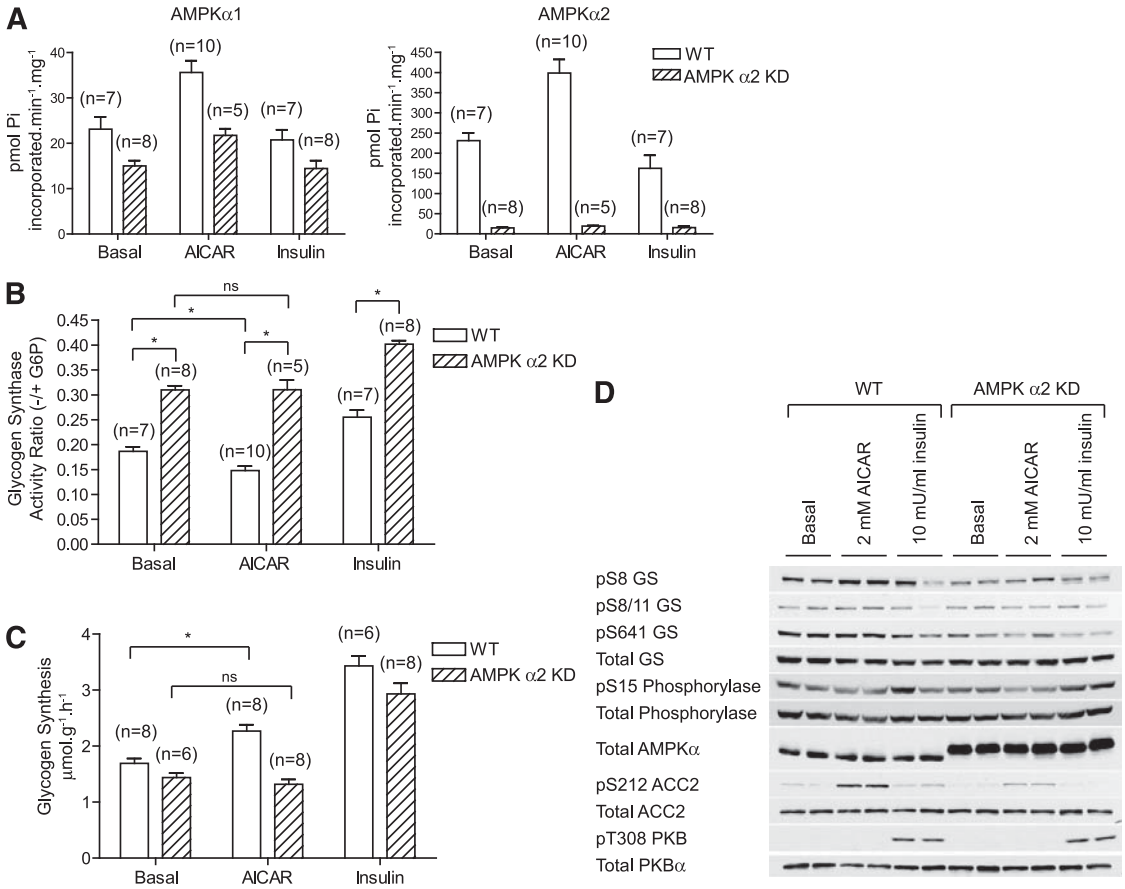

FIG. 6. AICAR-stimulated glycogen synthesis is AMPK-dependent. ***A*****: Isolated EDL muscles from 14- to 18-week-old wild-type (WT, □) or AMPKα2 KD mice (▨) were incubated with the indicated stimuli for 40 min, and AMPK activity was determined as described in Fig. 1.** ***B*****: Alternatively, muscles were incubated with the indicated stimuli for 40 min in KRB containing 5.5 mmol/L glucose and GS activity measured in tissue homogenates, as described in RESEARCH DESIGN AND METHODS.** ***C*****: Muscles were incubated with the indicated stimuli for 40 min in KRB containing D-[U-^{14}C]glucose (5.5 mmol/L), and the rate of glucose incorporation into glycogen was determined as described in RESEARCH DESIGN AND METHODS.** ***D*****: Muscles from WT and AMPKα2 KD mice were incubated with the indicated stimuli for 40 min and tissue lysates immunoblotted with the indicated antibodies. Results are presented as mean ± SEM from the indicated number (*n*) of mice. *$P < 0.05$.**

Abbott Laboratories has recently identified a direct and specific activator of AMPK, the thienopyridine A-769662, which is a useful tool to understand the physiologic consequences of AMPK activation in animals (36–38). However, Scott et al. (39) have demonstrated that this compound selectively activates β1-containing AMPK trimeric (αβγ) complexes, but not β2-complexes in cell-free assays. They further investigated the selectivity of A-769662 in vivo and showed that A-769662 failed to stimulate AMPK in AMPKβ1-deficient mouse tissues (39). The two β1-containing AMPK heterotrimers (α1β1γ1 and α2β1γ1), as well as activity associated with these complexes, appear to constitute only a small fraction of the total pool of AMPK trimeric complex/activity (i.e., <5%) in mouse skeletal muscle (40). A-769662 treatment resulted in only a modest activation of AMPK (37,39) and failed to promote AMPK-dependent increases in glucose uptake in isolated mouse skeletal muscles (40). Therefore, A-769662 would not be a suitable tool to study the effect of AMPK on glucose/glycogen metabolism in mouse skeletal muscle. As an alternative approach, we used a genetically engineered mouse model, AMPK KD, in which AMPK is inactivated by transgenic over-expression of a dominant inhibitory AMPKα2 mutant in muscle cells (6), and established that catalytic activity of AMPK is necessary to elicit AICAR-stimulated glycogen synthesis most likely by increased glucose transport and subsequent accumulation of intracellular G6P.

Consistent with previous studies, AICAR modestly decreased the muscle GS activity ratio (13,15), a measure of phospho-dependent activity, in cell-free assays in wild-type animals, and this effect was lost in AMPK KD mice. However, we failed to detect a consistent elevation in GS phosphorylation at site 2 (Ser8) or site 2 + 2a (Ser8 and Ser11) in EDL incubated with AICAR ex vivo. These antibodies have been stringently validated (24), so it would appear that the effect of AICAR on site 2 phosphorylation is modest (as evidenced by only ~10% decrease in GS

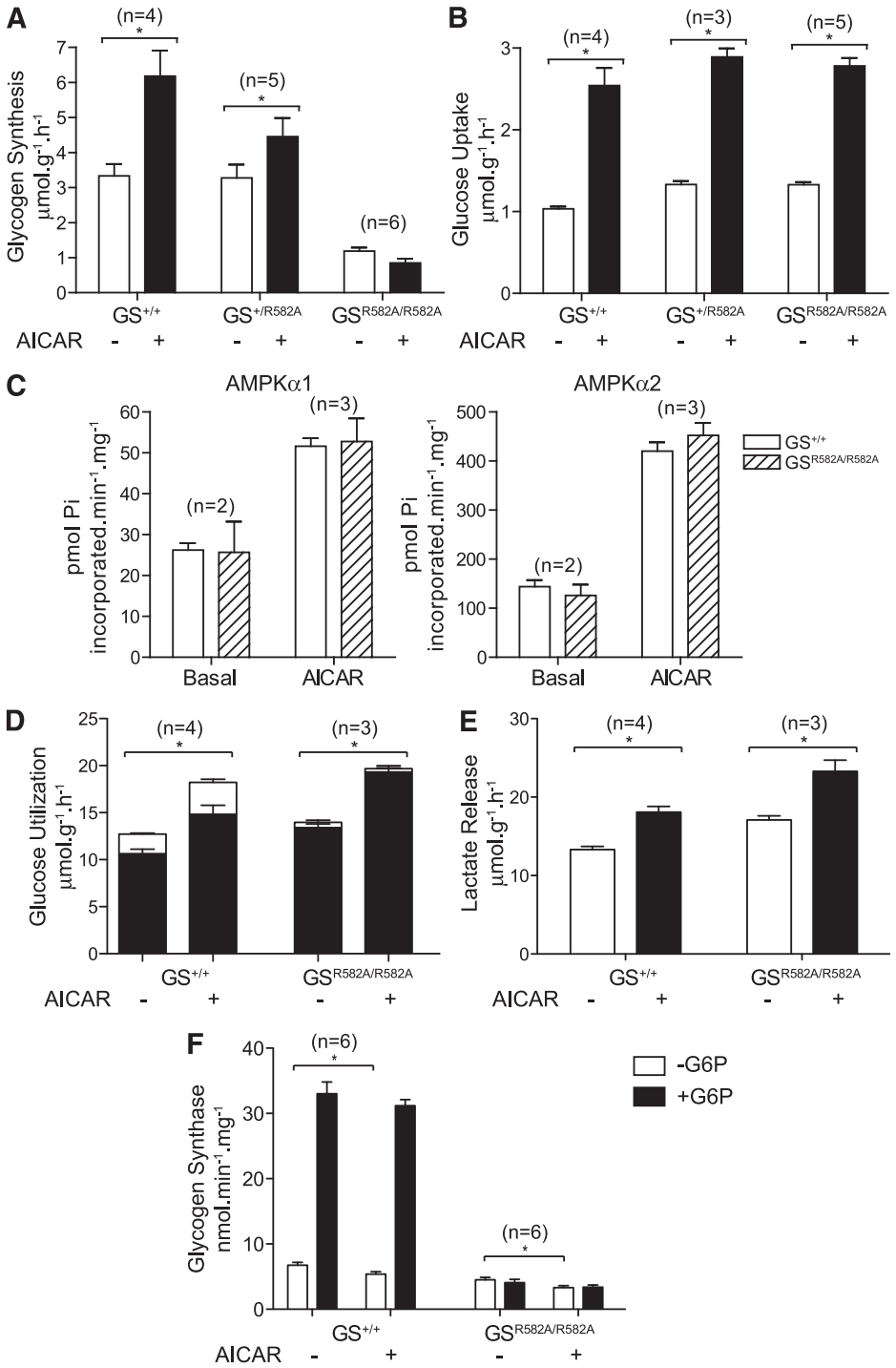

FIG. 7. AICAR-stimulated glycogen synthesis is dependent on allosteric activation of GS. GS R582A is unresponsive to allosteric activation by G6P (22). EDL muscles from the indicated genotypes were incubated with vehicle or 2 mmol/L AICAR in KRB containing D-[U-^{14}C]glucose (5.5 mmol/L)

activity), and in the current study, it failed to reach statistical significance.

This modest effect of AICAR on GS phosphorylation probably explains why a previous study failed to observe significant GS inhibition in skeletal muscle (8). A more robust effect of AICAR on GS activity and phosphorylation has been observed in rat skeletal muscle, although the reason for this is unclear (13). We tested if G6P directly affected GS phosphorylation by AMPK in response to AICAR. AMPK-mediated Ser8 phosphorylation of GS was not significantly altered in the presence of G6P in cell-free assays, although supraphysiologic concentrations of G6P (2 mmol/L) modestly reduced GS Ser8 phosphorylation by AMPK (Supplementary Fig. 2). Although we cannot completely rule out the possibility that AMPK inactivates GS through phosphorylation at other site(s), apart from Ser8, a previous in vitro phospho-mapping study did not identify additional phosphorylation sites (12). In support of this, there were no other obvious AMPK target site(s) (the typical consensus motif is ϕX[B, X)XX(Ser/Thr]XXXϕ, where ϕ is a hydrophobic residue, B is basic, and X is any residue) (1) on GS when motif scan analysis (Scansite, http://scansite.mit.edu/) was performed. Regardless of the mechanism, the modest inactivation of GS by AMPK was overridden by the allosteric stimulator, G6P, resulting in elevated GS activity in vivo as evidenced by an increase in [^{14}C]glucose incorporation into glycogen.

Gain-of-function mutation in AMPKγ1 (R70Q) (41) and naturally occurring mutations in γ3 (R200Q) originally identified in the Hampshire pig (*Sus scrofa domesticus*; *RN-*) (42), are known to promote marked glycogen accumulation in skeletal muscle. In addition, mutations in the AMPKγ2 subunit (encoded by the *PRKAG2* gene) have been implicated in the human cardiomyopathy, Wolff-Parkinson-White syndrome, which is characterized by ventricular pre-excitation, and in certain cases, cardiac hypertrophy (18). Notably, several genetic studies have revealed that γ2 mutations cause excess myocardial glycogen accumulation (43–45), which is hypothesized to cause conduction system abnormalities by unknown mechanism(s) (18,46). Interestingly, Luptak et al. (9) demonstrated that transgenic mice over-expressing one of the γ2 mutations (N488I) in cardiomyocytes displayed aberrant high activity of AMPK resulting in elevated intracellular [G6P] due to increased glucose uptake, which serves as both the carbon skeleton for glycogen synthesis and the allosteric stimulator of GS. It would be of major interest to cross AMPKγ2 mutant transgenic animals with G6P-resistant GS$^{R582A/R582A}$ knock-in mice and investigate if abnormal cardiac glycogen accumulation and the associated pathologies can be rescued.

In summary, we provide genetic evidence that AMPK-mediated glycogen synthesis occurs by increased activity of GS through its allosteric stimulator, G6P, and we propose the following model (Fig. 8): elevated glucose transport promoted by increased AMPK activity causes an accumulation of intracellular G6P. This leads to allosteric activation of GS, which overrides the inhibitory effect of AMPK on GS resulting in a net increase in GS activity and excess glycogen storage in muscle cells. Our work is of particular importance when considering AMPK as a target for treating metabolic disorders such as type 2 diabetes (47) because chronic/persistent activation of AMPK may have adverse consequences on cardiac function.

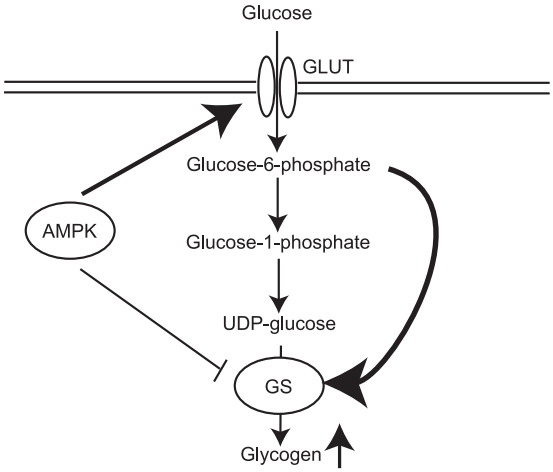

FIG. 8. Schematic summary shows the AMPK-mediated increase in muscle glycogen storage. Elevated glucose transport promoted by increased AMPK activity over an extended period, in the absence of a proportional increase in utilization of glucose, causes an accumulation of intracellular G6P. This leads to persistent allosteric activation of GS, which overrides the direct inhibitory effect of AMPK on GS and results in a net increase in GS activity and excess glycogen storage in muscle.

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R.W.H. designed and conducted most of the experiments, analyzed data, and wrote the manuscript. J.T.T. and J.F.P.W. conducted experiments, analyzed data, reviewed and edited the manuscript, and contributed to discussion.

for 40 min. *A*: Glycogen synthesis was assayed as described in RESEARCH DESIGN AND METHODS. *B*: Alternatively muscles were incubated with vehicle or 2 mmol/L AICAR and glucose transport assayed using 2-deoxy-[^{3}H]glucose as described in RESEARCH DESIGN AND METHODS. *C*: Basal or AICAR-stimulated muscles from GS$^{+/+}$ (□) and GS$^{R582A/R582A}$ (▨) mice were homogenized and assayed for AMPK activity as described in Fig. 1. *D*: Muscles were incubated with vehicle or 2 mmol/L AICAR in KRB containing [5-^{3}H]glucose for 40 min and the rates of glycolysis (■) and glycogenesis (□) determined as described in RESEARCH DESIGN AND METHODS. *E*: Alternatively, muscles were incubated with vehicle or 2 mmol/L AICAR in KRB/glucose for 40 min, and the rate of lactate release into the superfusate was determined enzymatically. *F*: Muscles were incubated with vehicle or 2 mmol/L AICAR in KRB/glucose for 40 min and GS activity was measured in muscle homogenates as described in RESEARCH DESIGN AND METHODS. Results are expressed as mean ± SEM for the indicated number (n) of animals. *$P < 0.05$ compared with basal.

K.S. designed and conducted experiments, analyzed data, wrote the manuscript, and also supervised the project.

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Successful immune tolerance induction to enzyme replacement therapy in CRIM-negative infantile Pompe disease

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Successful immune tolerance induction to enzyme replacement therapy in CRIM-negative infantile Pompe disease

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Purpose: Infantile Pompe disease resulting from a deficiency of lysosomal acid α-glucosidase (GAA) requires enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA). Cross-reactive immunologic material negative (CRIM-negative) Pompe patients develop high-titer antibody to the rhGAA and do poorly. We describe successful tolerance induction in CRIM-negative patients.

Methods: Two CRIM-negative patients with preexisting anti-GAA antibodies were treated therapeutically with rituximab, methotrexate, and gammaglobulins. Two additional CRIM-negative patients were treated prophylactically with a short course of rituximab and methotrexate, in parallel with initiating rhGAA.

Results: In both patients treated therapeutically, anti-rhGAA was eliminated after 3 and 19 months. All four patients are immune tolerant to rhGAA, off immune therapy, showing B-cell recovery while continuing to receive ERT at ages 36 and 56 months (therapeutic) and 18 and 35 months (prophylactic). All patients show clinical response to ERT, in stark contrast to the rapid deterioration of their nontolerized CRIM-negative counterparts.

Conclusion: The combination of rituximab with methotrexate ± intravenous gammaglobulins (IVIG) is an option for tolerance induction of CRIM-negative Pompe to ERT when instituted in the naïve setting or following antibody development. It should be considered in other conditions in which antibody response to the therapeutic protein elicits robust antibody response that interferes with product efficacy.

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Key Words: immune tolerance; methotrexate; Pompe disease; rituximab

INTRODUCTION

Infantile Pompe disease (OMIM# 232300) is a fatal disease resulting from a deficiency of lysosomal acid α-glucosidase (GAA).[1] Enzyme replacement therapy (ERT) with recombinant human acid α-glucosidase (rhGAA) is the sole disease-specific treatment currently available. Patients with two deleterious mutations and complete absence of GAA, as assessed by western blot, are classified as cross-reactive immunologic material negative (CRIM-negative). Patients with GAA protein detectable by western blot are classified as CRIM-positive.[2–4] Whereas the majority of CRIM-positive patients have sustained therapeutic responses to ERT, CRIM-negative patients almost uniformly do poorly, experiencing rapid clinical decline because of the development of sustained, high-titer antibodies to rhGAA.[4] CRIM-negative patients therefore serve as an excellent model to evaluate the impact of therapies aimed at immune tolerance.

We reported the first successful reversal of rhGAA antibodies in a CRIM-negative Pompe patient treated with rituximab, intravenous gammaglobulins (IVIG), and methotrexate.[5] We now report that this patient and an additional CRIM-negative patient treated similarly are indeed immune tolerant. Critically, such tolerance can be induced prophylactically, commencing with ERT, using a short rituximab with methotrexate regimen, thereby avoiding prolonged immune suppression. The two prophylactically treated patients and two therapeutically treated patients remain tolerant to continued administration of rhGAA, off of all immune therapy and with recovered B cells. All patients have achieved motor gains, in contrast to the relentless downhill course of nontolerant ERT-treated CRIM-negative patients. However, like some CRIM-positive patients, patients are left with residual deficiencies not reversible by ERT due to preexisting damage prior to the start of ERT.

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PATIENTS AND METHODS

This multinational collaborative effort received individual institutional review board or ethics committee approval. In all cases, motor assessment, cardiac assessment, and other clinical parameters were obtained from medical records. GAA mutation analysis was determined as previously described.[6] CRIM status was determined as previously described.[2] Briefly, cell lysates derived from the patient's fibroblasts were subjected to western blot analysis in a single laboratory with a polyclonal antibody that was made against human placental GAA, which recognizes all GAA protein forms. A patient is considered CRIM-negative if no GAA is detected in the western blot assay and the patients have deleterious mutations in the *GAA* gene. IgG antibodies to rhGAA were measured using enzyme-linked immunosorbent assays and confirmed using radioimmunoprecipitation, as previously described.[6] Urinary glucose tetrasaccharide (Glc_4) level was determined as the total hexose tetrasaccharide fraction in urine measured by high-pressure liquid chromatography with ultraviolet detection and electrospray ionization–tandem mass spectrometry as previously described[7]. Flow cytometry was used to assess CD19-positive (B cell) percentage, using standard techniques at each local institution.

rhGAA (Myozyme, Genzyme, Cambridge, MA) administered every 2 weeks was initiated after diagnosis of Pompe in all cases.[2,3] Dosing ranged from 20 to 40 mg/kg every 2 weeks.

Patients 1 and 2 were treated therapeutically by rituximab, methotrexate, and IVIG after the development of rhGAA antibodies, until antibodies were eliminated. Rituximab 375 mg/m^2/dose was given weekly for 4 weeks followed by maintenance dosing. Methotrexate 0.5 mg/kg weekly was given enterally, based on hematologic tolerance. IVIG 0.5 g/kg was given every 4 weeks (**Figure 1a,b**). Patients 3 and 4 were treated prophylactically with a short course of rituximab and methotrexate. Rituximab 375 mg/m^2/dose was administered weekly for 4 weeks (the first dose given 1 day before the first rhGAA administration), and methotrexate 0.4 mg/kg was given subcutaneously 3 times a week for 9–17 doses (**Figure 2a,b**).

RESULTS

Therapeutic Tolerance Induction for an Established Antibody

Patients 1 and 2 presented with hypotonia, cardiomyopathy, elevated creatinine phosphokinase (CPK) level, and elevated urine Glc_4 level at ages 5 weeks and 12 days, respectively. Both were confirmed as CRIM-negative Pompe patients (**Table 1**). rhGAA was initiated soon after diagnosis, at 7 weeks and 16 days of age, respectively (**Table 1**). IgG antibodies to rhGAA were detected after 4–6 weeks, escalating to maximum titers of 1:1,600 and 1:12,800, respectively. An immune tolerance regimen of rituximab, methotrexate, and IVIG was initiated as shown in **Figure 1**. Antibodies to rhGAA were fully eliminated 3 months after commencement of immune therapy in patient 1 (**Figure 1a**) and after 19 months in patient 2 (**Figure 1b**), although there was a rapid drop in titer following the first course. Patient 1 is tolerant to rhGAA after more than 4.5 years of ERT and patient 2 after 3 years of ERT. Both are off all immune therapy, with B-cell recovery (**Figure 1**). The immune tolerance regimen was very well tolerated with mild intermittent neutropenia requiring withholding methotrexate and only mild viral infections. Although patient 1 became ventilator dependent after 11 months of ERT, he continued to improve, now requiring ventilation only at night, whereas patient 2 was not ventilated (**Table 2**). All medications including rhGAA, rituximab, and IVIG were given at home to patient 1, under strict monitoring by skilled nursing staff. In both patients, cardiomyopathy rapidly resolved after initiating rhGAA (**Table 2**). Patients 1 and 2, diagnosed and treated before 2 months of age, had high baseline urinary Glc_4 values, which then decreased to less than those of CRIM-negative patients who did not receive immune tolerance therapy (**Figure 3**). Clinically, both patients continue to gain developmental milestones. Even though patient 1 has significant motor and speech deficits, he continues to improve, whereas patient 2 has only mild deficits (**Table 2**). Hearing tests were initially normal in both patients, but patient 1 developed mild conductive hearing loss in the middle-to-high frequencies at the age of 56 months, and patient 2 required pressure-equalizing tubes at the age of 26 months. At the age of 54 months, magnetic resonance imaging (MRI) of the brain of patient 1 revealed new, extensive deep white matter changes, sparing the subcortical U-fibers (**Figure 4**), whereas MRI of the brain of patient 2 (age 31 months) was normal (**Table 2**). Patient 1 is receiving rhGAA every 2 weeks to date, patient 2 received rhGAA every 2 weeks, but after 29 months of ERT, rhGAA frequency was increased to every week due to increased creatinine phosphokinase (CPK) levels, progression of ptosis, increased fatigue, and decreased stamina, all of which have since improved.

Prophylactic Tolerance Induction

Patients 3 and 4 presented with hypotonia, cardiomyopathy, and elevated creatinine phosphokinase (CPK) level at the ages of 8 and 10 weeks, respectively. They were confirmed as CRIM-negative Pompe patients (**Table 1**) and received the tolerance-induction regimen simultaneously with rhGAA at 15–16 weeks of age (**Table 1**, **Figure 2**). Patient 3 also received one dose of IVIG at week 4 due to a febrile illness. Both patients continued to receive rhGAA without further immune therapy. Patient 3 developed a transient anti-rhGAA antibody response, which reached a maximal titer of 1:1,600 and spontaneously declined to 1:200 after 24 months on ERT (**Figure 2a**) without additional immune tolerance therapy. In patient 3, who at diagnosis had significant cardiac involvement with poor function, cardiomyopathy improved after 14 months of ERT, both in function and wall thickness, but did not completely resolve (**Table 2**). Ventilation was not required, and she continues to gain developmental milestones (**Table 2**). Commensurate with her excellent clinical course, Glc_4 levels of patient 3 remain lower than those of nontolerized CRIM-negative patients (**Figure 3**). Patient 4 was persistently antibody negative (**Figure 2b**) and required only transient ventilation for 3 days at diagnosis. At diagnosis, he had significant cardiac involvement—dilated cardiomyopathy with very poor function. Although the LV mass improved,

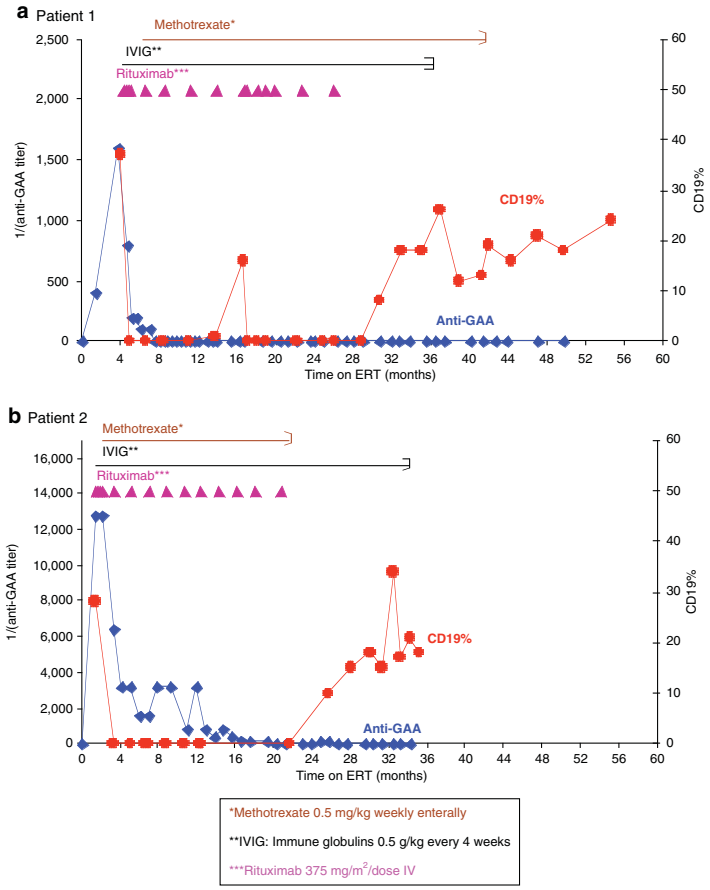

Figure 1 Therapeutic-treated patients with antibody to ERT. Tolerance-induction regimen, with rituximab, methotrexate, and intravenous gammaglobulins (IVIG) showing antibody levels and B-cell percentage for patients treated therapeutically for an established antibody response. Both patients achieved tolerance to ongoing enzyme replacement therapy, and are off immune therapy, with negative antibody titer showing B-cell recovery. (**a**) Patient 1. (**b**) Patient 2. ERT, enzyme replacement therapy.

the function to date has not changed (Table 2). He attained developmental milestones, but a parainfluenza infection at the age of 14 months resulted in neuromotor regression. His overall worse disease status is reflected in high Glc_4 levels, which are currently comparable to those of nontolerized CRIM-negative patients (Figure 3). Patient 3 had normal MRI of the brain at the age of 17 months and an inconclusive hearing test at 12 months (which was not repeated), whereas patient 4 did not have an MRI or a formal hearing test (Table 2).

DISCUSSION

The clinical use of therapeutic enzymes has expanded rapidly in the past decade for a variety of disorders, with ERTs now available for several previously untreatable diseases. Therapeutic enzymes are potentially immunogenic, evoking antibody responses that may be without overt clinical significance or may lead to hypersensitivity reactions, decreased bioavailability, or decreased efficacy.[8,9] Antibody-mediated abrogation of efficacy of the therapeutic proteins has been described in patients with other enzyme- and factor-deficiency states such as mucopolysaccharidosis types I, II, and VI, Fabry disease, and severe hemophilia A and B.[10–14]

Most Pompe disease patients develop antibodies to ERT, but with dramatically different outcomes, depending on CRIM status. Antibody titers in the majority of CRIM-positive patients diminish over time and do not appear to neutralize efficacy, leaving patients with a favorable clinical response. In contrast, CRIM-negative patients and a subset of CRIM-positive patients mount high-titer and sustained antibody responses

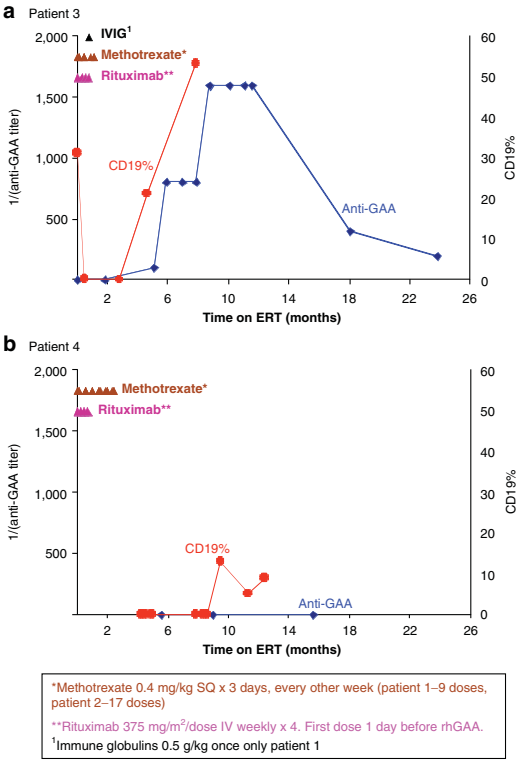

Figure 2 Prophylactic-treated patients. Tolerance-induction regimen, antibody levels, and B-cell percentage for patients treated prophylactically in the naïve setting with rituximab and methotrexate. Both patients are tolerant to ongoing enzyme replacement therapy, off immune therapy with B-cell recovery. (**a**) Patient 3 with remains with low-titer (1:200) antibody level, similar to CRIM-positive patients. (**b**) Patient 4 with negative antibody titer.

that neutralize enzyme uptake into cells with resulting poor outcome: death or invasive ventilation in patients by the age of 27 months.[4,15] Therefore, there is clearly a need for immune tolerance induction to ERT in CRIM-negative Pompe patients and those CRIM-positive patients who are at risk for development of high sustained antibody titers.

We have previously described patient 1,[5] who was treated for an established antibody response, and now confirm his immune tolerance to the foreign protein. Remarkably, this patient and an additional patient treated for established antibody are off all immune suppressive therapy, have B-cell recovery, and are tolerant to rhGAA. Both patients had no significant toxicity or increased infections from this regimen, with the exception of transient leukopenia from methotrexate. Importantly, both patients began immune tolerance therapy relatively early after detection of an antibody response to rhGAA prior to development of a very high titer and sustained response. It is certainly possible that patients with very high persistent antibody titers (more than 1:51,200) may not respond to this regimen.[15]

To assess whether a short course of immune tolerance induction therapy at commencement of ERT (thus avoiding prolonged immune suppression) would confer lasting tolerance, two additional CRIM-negative patients were treated prophylactically with a short regimen of rituximab and methotrexate essentially without IVIG. Remarkably, both patients display ongoing tolerance to ERT: patient 3 developed a transient antibody response that declined over time, similar to that seen in CRIM-positive patients, tolerating ERT at the age of 35 months with clinical gains. Patient 4 did not develop rhGAA antibodies but has a dilated cardiomyopathy and neuromotor deficits.

This regimen was chosen for tolerance induction in both therapeutic and prophylactic settings on the basis of data from clinical trials and experimental data. Rituximab has been used successfully in immune-mediated diseases, such as systemic lupus erythematosus[16] as well as hemophilia patients with high-titer inhibitors.[17] In patients with severe factor VIII deficiency with high-titer inhibitors, rituximab induced tolerance to therapeutic factor VIII with a variable degree of success.[17–20] The mechanism by which rituximab induces tolerance is unknown but, in some circumstances, appears to be related to restoration of T regulatory cells.[21] Regardless of mechanism, the success of the approach suggests that B cells are critical in the induction and maintenance of the immune response.

Interestingly, a recently reported CRIM-negative patient received another monoclonal agent, omalizumab, an anti-IgE, to prevent anaphylaxis to rhGAA. Strikingly, this patient has very low-titer IgG against rhGAA (1:400) after 44 months of ERT.[22] This suggests some heterogeneity among CRIM-negative patients in the nature of the immune response to rhGAA as well as a novel use of anti-IgE.

One of the concerns with rituximab therapy is the profound B-cell suppression leading to decreased serum Ig levels and the heightened potential for adverse outcomes. Patient 1 failed to respond to routine vaccination administered in the treatment period and even after B-cell recovery (data not shown), suggesting the potency of this regimen. It suggests that following successful treatment, vaccination response should be monitored. To decrease infectious complications, both patients who had received prolonged rituximab therapy were placed on chronic IVIG. Additionally, it is well known that IVIG is also an immunomodulatory agent used extensively in the setting of autoimmune disease[23,24]; and thus, it is probable that the IVIG had dual effects in our patients: contributing to immune modulation and protecting against infectious agents.

Prolonged rituximab therapy with B-cell suppression has been associated with the development of progressive multifocal leukoencephalopathy (PML).[25] Patient 1 developed changes of the brain affecting the deep white matter (detected on MRI; **Figure 4**), which are clearly different from what is seen in progressive multifocal leukoencephalopathy that affect the subcortical area.[25] Additionally, this patient shows slow progressive neurologic improvement rather than the clinical deterioration seen in progressive multifocal leukoencephalopathy.[25] Thus this patient does not have PML. Since these MRI changes are similar to a

Table 1 Baseline and immunologic data for patients treated therapeutically (patients 1 and 2) for an established antibody response as well as patients treated prophylactically (patients 3 and 4) in parallel to starting enzyme replacement therapy

	Therapeutic tolerance induction		Prophylactic tolerance induction	
	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
Age of diagnosis	5 weeks	12 days	8 weeks	10 weeks
Sex	M	F	F	M
Race	African American	Caucasian	Caucasian	Caucasian
Consanguinity	No	No	Yes	No
GAA mutation	Homozygous c.2560C>T	c.1128-1129delinsC/ c.2237G>A	Homozygous c.341insT	c.1548G>A and c.525delT
GAA activity	Absent	Absent	Absent	Absent
Initiation of ERT	7 weeks	16 days	16 weeks	15 weeks
Seroconversion	6 weeks	4 weeks	38 weeks	None
Onset of immune tolerance after ERT	18 weeks	6 weeks	0 weeks	0 weeks
Current immune therapy	None	None	None	None
Anti-rhGAA titer				
Peak titer (time after ERT)	1:1,600 (4 months)	1:12,800 (1 month)	1:1,600 (9 months)	Negative
Time to antibody elimination	3 months	19 months	Not eliminated	N/A
Last titer	Negative	Negative	1:200	Negative
B-cell recovery	Yes	Yes	Yes	Yes
Urinary Glc_4 level (mmol/mol CN)				
Baseline	26	23	NA	NA
6 months	39	20	NA	29
12 months	48	10	NA	38
Last	47 (53 months)	17 (35 months)	21 (33 months)	69 (22 months)

ERT, enzyme replacement therapy; GAA, acid α-glucosidase; Glc4, glucose tetrasaccharide; rhGAA, recombinant human GAA.

previously described CRIM-negative patient, they may reflect the natural history of CRIM-negative Pompe disease.[22]

Finally, methotrexate was added to the regimen because it eliminates dividing lymphocytes, theoretically eliminating rhGAA-specific T cells and residual B cells. Methotrexate also minimized antibody to rhGAA when used as a sole agent in a mouse model of Pompe disease.[26]

Remarkably, all tolerized patients showed motor improvement, in stark contrast to ongoing deterioration of CRIM-negative patients, confirming the role of the immune response in abrogating effectiveness of ERT.[4] Nonetheless, patients were left with residual deficiencies either due to irreversible changes or due to the inability of rhGAA to fully address disease manifestations. Patient 3 is left with mild cardiomyopathy, and patient 4 continues to have significant residual dilated cardiomyopathy despite immune tolerance to ERT. Both patients were diagnosed and started therapy at a later age, and it is possible that irreversible myocardial damage occurred prior to the start of ERT. Patient 1, at 56 months, has shown a clinical response to ERT and continues to make motor and developmental progress; however, he has motor and speech delays. His pulmonary status has improved from full-time ventilation to requiring ventilator support only at night. The poorest outcome was in patient 4, who at the start of ERT already had significant motor involvement. Following appreciable motor improvement, this patient had motor deterioration with elevated urinary Glc_4 level after a parainfluenza infection, thus illustrating the fragility of such patients. This outcome suggests that early identification of infantile Pompe disease, ideally via newborn screening, may abrogate these irreversible changes. It is critical to note that even patients 1 and 4, who had significant deficits, continue to have neuromotor improvement in contrast to the ongoing deterioration of CRIM-negative patients with high-titer antibody.[4,15]

The deficits seen in patients 1 and 4 and the elevated Glc_4 level of patient 4 may be secondary to basic limitations of ERT in Pompe disease. These include the high levels of rhGAA required to achieve efficacy—approximately 30-fold to 100-fold greater than doses for ERT in other lysosomal disorders[27]—and the well-recognized variability of response by skeletal muscle. Potential factors involved in this variability include a lower

Table 2 Outcome clinical data

	Therapeutic tolerance induction		Prophylactic tolerance induction	
	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
Current age	56 months	36 months	35 months	22 months
rhGAA	20 mg/kg every 2 weeks	40 mg/kg every week*	20 mg/kg every 2 weeks	20 mg/kg every 2 weeks
Ventilation	From 13 months to only at night	None	None	3 days at diagnosis
LVMI (gm/m$^{2.7}$)				
Baseline	202	117	NA	NA
6 months	75	59**	NA	389
12 months	60	NA	NA	273
Last	31 (48 months)	44 (30 months)	NA	194 (15 months)
FS				
Baseline	40%	36%	18%	7%
6 months	42%	35%**	15%	10%
12 months	64%	NA	26%	4%
Last	38% (48 months)	35% (30 months)	25% (27 months)	11% (15 months)
Cardiomyopathy	Resolved (6 months)	Resolved (3 months)	Improved (14 months)	Ongoing
Neuromotor status	Scoots in sitting for independent floor mobility; pulls to stand independently, maintains standing with moderate assistance; fine motor skills advancing; communication impaired by poor intelligibility.	Age-appropriate gross motor skills, with isolated residual weakness, decreased core strength, myopathic facies, ptosis; no difficulty swallowing. Mild speech delay; low average overall cognitive abilities: gross, fine motor and visual reception—average, language ability—below average.	Walks and runs independently. No difficulty swallowing. Speaks, but with hypernasal speech as is seen in other children who survive infantile Pompe disease.	Prop-sit independently, sits briefly without hand support, rolls from supine to side lying, bears weight through lower extremities in supported standing. Swallows solid food and drools less than previously. Age-appropriate hand function and speech.
Sits independently	Yes	Yes	Yes	Yes
Bears weight through lower extremities	Yes	Yes	Yes	Yes
Independently ambulatory	No	Yes	Yes	No
MRI of brain (age in months)	New deep white matter changes (age 54 months)	Normal (age 31 months)	Normal (age 17 months)	Not done
Hearing test	Mild conductive hearing loss—middle to high frequencies (age 56 months)	PE tubes (age 26 months)	Inconclusive (age 12 months)	Not done

ERT, enzyme replacement therapy FS, fractional shortening by m-mode echocardiography; LVMI, left ventricular mass index per height$^{2.7}$ (gm/m$^{2.7}$); MRI, magnetic resonance imaging; NA, not available; PE tubes, pressure-equalizing tubes; rhGAA, recombinant human acid α-glucosidase; CPK, creatinine phosphokinase.

*Patient 2 received rhGAA 20 mg/kg, which was changed to 30 mg/kg after 17 months of ERT and then to 40 mg/kg after 23 months. Frequency was increased to weekly 40 mg/kg after 29 months of ERT due to increased CPK levels, progression of ptosis, increased fatigue, and decreased stamina, all of which have since improved.

**Patient 2 echocardiography data are for 9 months of ERT. No data for 6 or 12 months.

number of mannose-6-phosphate receptors (the principal receptors for enzyme uptake and lysosomal targeting) in skeletal muscle in comparison to the heart, inefficient targeting to skeletal muscle, accumulated muscle and lysosomal damage, and resistance to correction by type II myofibers.

In conclusion, strategic use of rituximab and methotrexate with or without IVIG has been effective in prophylaxis against an immune response and in reversing the immune response in CRIM-negative infantile Pompe patients treated with ERT. An ongoing study in treatment-naïve CRIM-negative patients using a similar prophylactic protocol is accruing patients (http://clinicaltrials.gov, NCT00701129). Rituximab and methotrexate with IVIG should be used in CRIM-negative patients with antibodies to rhGAA and should also be considered for the rare CRIM-positive Pompe patients at risk for a high-titer sustained immune response.

This successful tolerance induction to rhGAA in CRIM-negative Pompe patients will allow for better understanding of the neurological and muscular challenges facing CRIM-negative patients who survive longer tolerating the ERT. It allows us to compare tolerant CRIM-negative versus CRIM-positive patients for differences, if any, in the central nervous system and other organs.

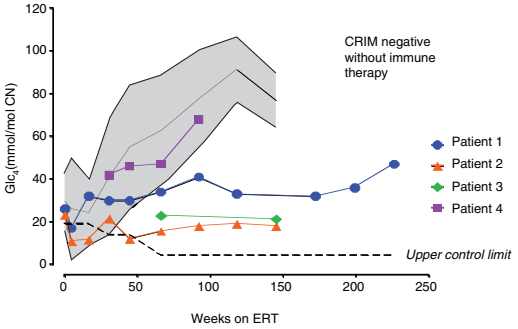

Figure 3 Change in urinary glucose tetrasaccharide (Glc_4) biomarker concentration on enzyme replacement therapy for the four patients treated by immune therapy. Comparative data for CRIM-negative patients without immune therapy (n = 8, ± SD) are displayed in gray. Mean value of Glc_4 over different time periods (bins) was calculated for each patient, and median value for each bin was used to plot the time point. Upper limit of normal controls is represented by dashed line, which is ≤4.4 mmol/mol CN for more than 1 year of age. Therapeutically treated patient 1 Glc_4 levels (mean ± SD: 31 ± 13 mmol/mol CN, 26 measurements) and patient 2 Glc_4 levels (16 ± 6 mmol/mol CN, 40 measurements) remained lower than those of nontreated CRIM-negative patients. Similarly, prophylactically treated patient 3 Glc_4 levels remained lower than those of nontreated CRIM-negative patients (21, 23 mmol/mol CN). In contrast, patient 4 had levels that were comparable to those of the nontreated CRIM-negative group (50 ± 14 mmol/mol CN, 8 measurements). Note that for patients 3 and 4, last urine samples for Glc_4 were available for later time points than anti-acid α-glucosidase (GAA) antibody measurement depicted in Figure 2.

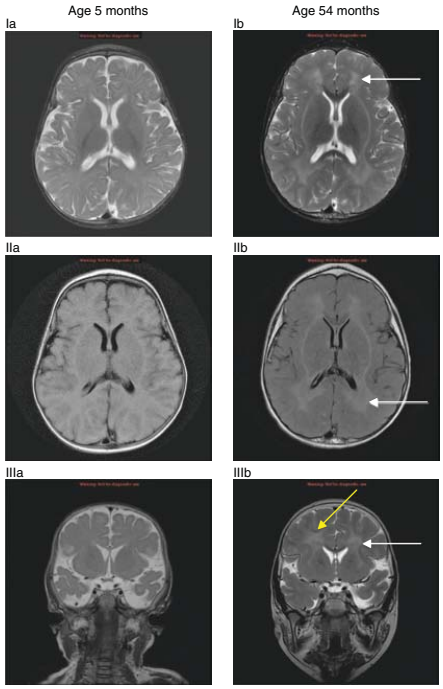

Figure 4 Long-term changes on brain magnetic resonance imaging (MRI) of patient 1. The images on the left, taken at age 5 months, show normal myelination for age. The images on the right, at age 54 months (4.5 years) show development of T2 prolongation within the deep white matter (white arrows) in the periventricular white matter, centrum semiovale, and external capsule, sparing the subcortical U-fibers (yellow arrow) as well as the internal capsule. I: T2 axial scans at ages (Ia) 5 months and (Ib) 54 months; II: T2 fast low-angle inversion recovery (FLAIR) images; III: T2 coronal scans.

This tolerance-inducing strategy might also be more broadly applicable to other conditions treated with therapeutic proteins such as other lysosomal storage diseases, severe hemophilia A and B, and other conditions in which a foreign protein elicits robust antibody responses that interfere with product efficacy. These lessons may also be important for gene therapy approaches, which may be functionally limited by immune responses.

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II - Guías actualizadas

GUÍAS INFORMATIVAS DE LA AEEG

• Guía Informativa para la Glucogenosis Tipo I. Enfermedad de von Gierke.

• Guía Informativa para la Glucogenosis Tipo II. Enfermedad de Pompe.

• Guía Informativa para la Glucogenosis Tipo V. Enfermedad de McArdle.

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AEEG
Asociación Española de Enfermos de Glucogenosis

La AEEG está constituida por pacientes y familias de pacientes afectados por los distintos tipos de glucogenosis, así como por personal sanitario con interés en el tratamiento de estas enfermedades.

La AEEG persigue los siguientes objetivos:

• Difundir información médica entre pacientes en un lenguaje comprensible.

• Facilitar el acceso de pacientes y del personal sanitario a fuentes de información y grupos de apoyo.

• Promover el contacto entre pacientes, médicos y las autoridades sanitarias.

• Difundir entre los enfermos y la comunidad médica los últimos avances científicos en el tratamiento de los distintos tipos de glucogenosis.

• Ayudar a la financiación de estudios y proyectos de investigación que promuevan un mejor conocimiento de estas enfermedades y el desarrollo de nuevas terapias para su tratamiento.

• Organizar congresos y reuniones que faciliten el contacto entre el personal sanitario y los investigadores interesados en el tratamiento de las distintas glucogenosis.

• Publicar guías informativas para su difusión entre los pacientes y la comunidad médica.

• Promover el apoyo mutuo y el asociacionismo entre pacientes y familias de pacientes afectados por los distintos tipos de glucogenosis, así como la colaboración con otras asociaciones centradas en la lucha contra las enfermedades raras.

GUÍA INFORMATIVA PARA LA GLUCOGENOSIS TIPO IX (DEFICIENCIA DE FOSFORILASA KINASA)

6ª edición

Laura Castells Molines

Leonor Fernández Marcos

Alberto Molares Vila

Abril de 2013

¿QUÉ ES LA GLUCOGENOSIS TIPO IX?

La glucogenosis tipo IX es una enfermedad metabólica hereditaria, consistente en una deficiencia congénita de la enzima Fosforilasa b Kinasa (FBK). Esta enzima tiene como función activar a otra enzima: la fosforilasa, la cual juega un papel fundamental en el metabolismo del glucógeno al regular la glucogenólisis en diversos tejidos del organismo. Una ausencia o deficiencia de la FBK provoca la inactividad de la fosforilasa, situación que puede traducirse en una acumulación de glucógeno, principalmente en el hígado y en el tejido muscular.

Esta patología está incluida, por tanto, dentro del grupo de glucogenosis que provocan alteraciones en el sistema de la fosforilasa. La fosforilasa tiene como función la obtención de glucosa a partir de las reservas de glucógeno mediante su fosforilización. Las enzimas implicadas en la activación de la fosforilasa son la adenilato ciclasa, la proteína cinasa y la fosforilasa b kinasa (también denominada fosforilasa b cinasa), dando lugar las deficiencias en cada una de ellas a distintos tipos de glucogenosis. Inicialmente, la deficiencia en la Fosforilasa b Kinasa fue clasificada como un subtipo de la glucogenosis tipo VI, también conocida como enfermedad de Hers o déficit de fosforilasa [1-2]. Sin embargo, desde mediado de la década de los setenta la deficiencia en FBK se categoriza individualmente como glucogenosis tipo IX [3]. Aún así, persisten algunos autores que no acaban de aceptar esta ordenación numérica [4].

La glucogenosis tipo IX se caracteriza por ser una de las formas más benignas de glucogenosis, pues provoca síntomas sólo durante la infancia y adolescencia. Con la edad, las secuelas clínicas y bioquímicas propias de la enfermedad tienden a remitir gradualmente, y la mayor parte de los adultos son asintomáticos.

Entrada nº 306000 en McKusick´s catalogue: Mendelian Inheritance in Man (OMIM) [5].

SINÓNIMOS

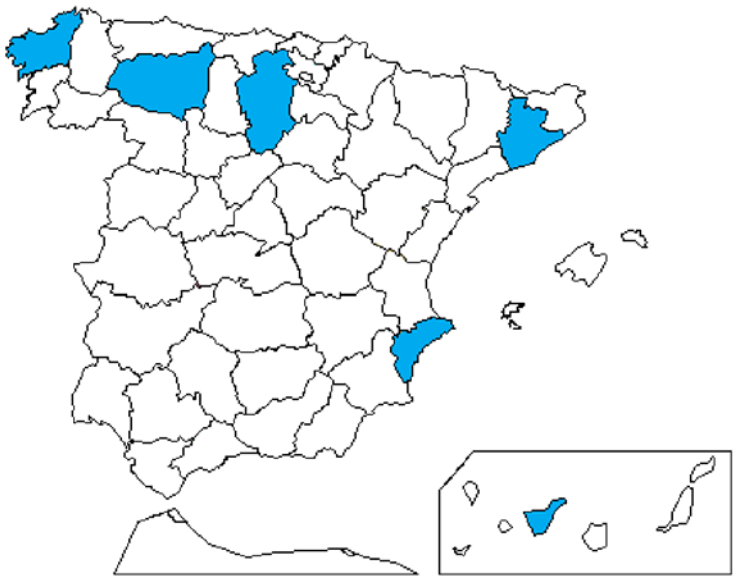
Deficiencia de Fosforilasa Kinasa
Deficiencia de Fosforilasa b Kinasa (FBK)
Glucogenosis Hepática Benigna

La glucogenosis tipo IX puede incluirse en cualquiera de las siguientes categorías:

- Glucogenosis.
- Enfermedades metabólicas.
- Enfermedades de depósito.
- Enfermedades genéticas.
- Enfermedades raras.

INCIDENCIA

Diversos estudios confirman que esta patología podría ser una de las glucogenosis más comunes; sin embargo, debido principalmente al buen pronóstico de la enfermedad, en muchos casos puede pasar inadvertida y no ser diagnosticada. Esto puede explicar que en las asociaciones de enfermos suelan ser minoritarios los afectados por este tipo de glucogenosis.



*Distribución geográfica de la enfermedad de FBK en España según datos de la AEEG. No obstante, es muy posible que la distribución real de la enfermedad sea más amplia, ya que aquellos pacientes residentes en regiones sin médicos con experiencia es más que probable que no estén diagnosticados.

SUBTIPOS CLÍNICOS

La Fosforilasa B Kinasa es una proteína compleja, ya que tiene una naturaleza de enzima tetramérica, al estar divida en cuatro subunidades proteicas distintas (Alpha, Beta, Gamma y Delta), cada una de ellas determinada por un cromosoma diferente. Las alteraciones en cada una de estas subunidades dan lugar a varios subtipos de la glucogenosis tipo IX, cada uno caracterizado por afectar a tejidos distintos y por tener un modo propio de herencia.

Los tres subtipos más comunes se clasifican como glucogenosis tipo IX-a, IX-b y IX-c, y están ligados, respectivamente, a alteraciones en las subunidades proteicas Beta (cromosoma 16), Alpha (cromosoma X) y Gamma (cromosoma 7). Las mutaciones causantes de alteraciones en la subunidad Delta están ligadas a la regulación del calcio, pero no tienen secuelas clínicas. Todos los subtipos producen afectación durante la infancia, aunque los síntomas, en la mayor parte de los casos, tienden a remitir a lo largo de la vida del paciente.

• Glucogenosis tipo IX-a, o déficit autosómico de Fosforilasa b Kinasa hepática.
Sigue un patrón de herencia autosómico recesivo, al estar localizado el gen responsable de la mutación en el cromosoma 16q12. De la misma forma que el subtipo IX-c, puede afectar por igual a hembras y a varones. El subtipo IX-a se caracteriza por remitirse exclusivamente al hígado, no provocando afectación alguna del músculo esquelético.

• Glucogenosis tipo IX-b, o déficit de Fosforilasa b Kinasa hepática ligada al cromosoma X (XLG).
Es probablemente el subtipo más frecuente [6]. Sigue un patrón de herencia ligado al sexo, y afecta normalmente sólo a varones. No hay afectación del músculo esquelético, al igual que en el tipo IX-a, siendo estas dos variedades clínicamente indistinguibles entre sí. El síntoma más frecuente es la hepatomegalia, que puede ir acompañado, entre otras manifestaciones adicionales, por una hipoglucemia moderada, retrasos en el crecimiento, hipercolesterolemia, hipertrigliceridemia e hipercetosis. Al mejorar los síntomas con la edad, la mayor parte de los adultos tienen una estatura normal y no sufren dolencias hepáticas.

La variedad XLG se divide, asimismo, en dos subtipos: XLG I, con una deficiencia en la actividad de la fosforilasa kinasa en sangre y en el hígado; y XLG II, con una actividad normal en sangre pero variable en el hígado. Ambos subtipos son causados por mutaciones en genes distintos [7-9].

La deficiencia de fosforilasa kinasa en músculo (glucogenosis muscular) está catalogada como Glucogenosis tipo VIII, de la cual la AEEG no tiene constancia de personas afectadas en la actualidad, probablemente debido a que puede ser subdiagnosticada en la mayoría de los casos.

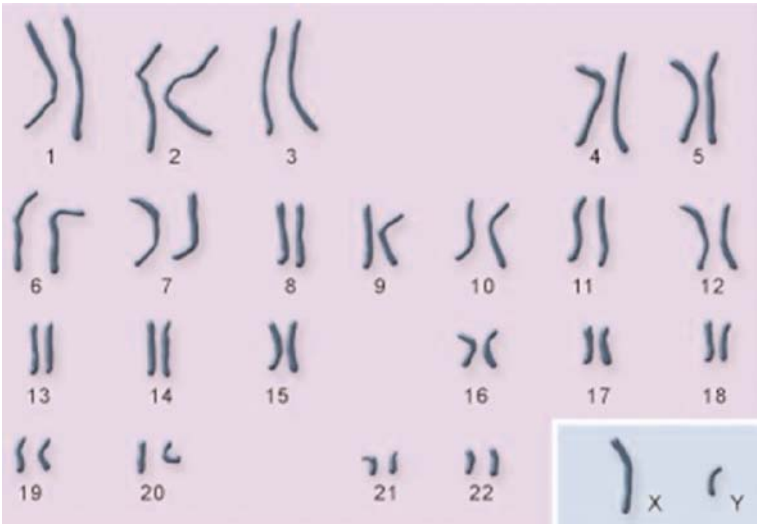
• Glucogenosis tipo IX-c, o déficit autosómico de Fosforilasa b Kinasa hepática y muscular.
Con una transmisión autosómica recesiva, al estar ligado al cromosoma 7p12, este déficit enzimático afecta al hígado y también al músculo. Los síntomas predominantes durante la niñez son hepatomegalia y retraso en el crecimiento.

Algunos pacientes pueden presentar también hipotonía muscular que normalmente suele ser moderada, aunque en algunos casos puede ser severa e ir acompañada de contracturas. Se han descrito también casos en los que este subtipo produce miocardiopatía, aunque esto es extremadamente raro.

PATRÓN DE HERENCIA LIGADO AL CROMOSOMA

Herencia ligada al sexo en glucogenosis tipo IX

Tenemos 23 pares de cromosomas, es decir 46 en total. Durante la fecundación, el óvulo que tiene 23 cromosomas, se fusiona con un espermatozoide, que también tiene 23 cromosomas, originando un feto con 46 cromosomas. Del total de cromosomas, 22 pares son los llamados autosomas, y un par son los cromosomas sexuales. Los cromosomas sexuales son el X y el Y, y son los que determinan nuestro sexo. Una mujer tendrá 2 cromosomas X (XX) y un hombre tendrá un cromosoma X y un cromosoma Y (XY).



La glucogenosis tipo IX tiene un patrón de herencia ligado al cromosoma X. Esto quiere decir que afecta al par de cromosomas sexuales, es decir, que la enzima deficitaria que produce la glucogenosis tipo IX es producto de un gen que se localiza en el cromosoma X. Así, dependiendo del sexo puede tener un patrón u otro.

Como las mujeres tienen 2 cromosomas X (XX), pueden darse 3 posibilidades:

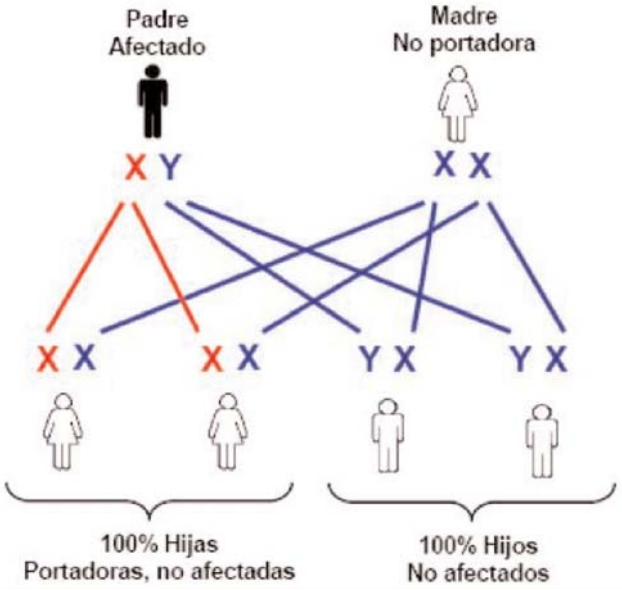
1. Mujeres sanas ◊ Si tienen los 2 cromosomas X sanos (XX).
2. Mujeres portadoras ◊ Si uno de los cromosomas está sano pero el otro tiene la alteración (XX).
3. Mujeres afectadas ◊ Las 2 copias del cromosoma X están alteradas (XX).

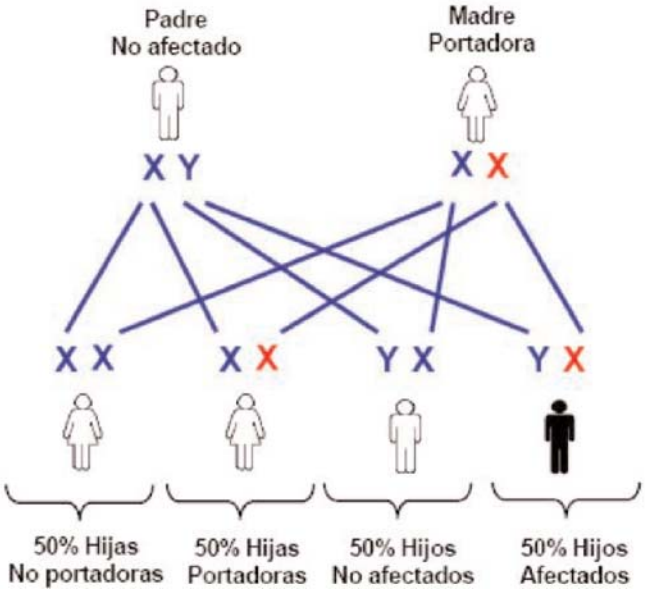
En el caso de los varones, al tener un cromosoma X y un cromosoma Y, solo se pueden dar 2 posibilidades:

1. Varón sano ◊ Tiene su cromosomas X sano (XY).
2. Varón afectado ◊ La copia del cromosoma X está alterada (XY).

En el patrón de herencia ligado al cromosoma X nunca hay varones portadores, ya que al tener solo un cromosoma X, se expresa completamente. En las mujeres puede haber portadoras ya que al tener solo un cromosoma X afectado, el otro puede expresarse completamente y corregir la deficiencia.

Podemos apreciar en las siguientes figuras 2 ejemplos de descendencia entre un padre afectado y una madre sana y entre un padre sano y una madre portadora:





SÍNTOMAS DE LA GLUCOGENOSIS TIPO IX

Aunque cada paciente puede presentar peculiaridades propias, dependiendo del subtipo de la enfermedad que sufra y de su grado efectivo de deficiencia enzimática, puede afirmarse que los síntomas más característicos de la enfermedad durante la infancia son [10-12]:

• **Hepatomegalia.** El principal síntoma y común entre estos casos es la distensión abdominal.

Esta hepatomegalia suele ser masiva en una primera fase temprana de la vida, pero va cediendo gradualmente, tendiendo a desaparecer o a ser muy leve en la adolescencia y en la vida adulta. En algunos casos puede ir acompañada por un agrandamiento del bazo.

• **Hipoglucemia.** Suele ser leve en los casos que aparece, y provoca valores bajos de glucosa en sangre en periodos de ayuno. Aun así, existe una respuesta normal de la glucosa de la sangre al glucagón.

Existen casos conocidos, en los que han manifestado vómitos por las mañanas debido a esta hipoglucemia, con un fuerte olor a acetona.

• **Menor estatura.** Suelen estar algo por debajo de la media de sus compañeros, pero no por ello son los más bajos; en la mayoría de los casos su altura y peso se desarrollan con gran equilibrio y normalidad de acuerdo su edad.
Sin embargo, la mayor parte de los pacientes adquieren una talla normal con el curso de los años.

• **Retraso en la pubertad.**

• **Niveles por encima de lo normal de las enzimas hepáticas.**

• **Niveles de colesterol y triglicéridos ligeramente elevados.**

• **Hipotonía muscular cuando hay déficit de la enzima en el músculo.** Cuando se presenta suele ser leve, aunque generalizada.

• **Desarrollo psicomotor.** Existen diferentes experiencias, hay casos en que el niño no presenta ni manifiesta ningún problema a este nivel, pero hay otros en que si se aprecia algún retraso al respecto. Hay niños que desde muy pequeños han practicado algún deporte sin ningún tipo de limitación, al principio sus padres, como es lógico, se preocupaban por cómo actuaría el niño a medida de que se fuera cansando, teniendo a mano alguna bebida o alimento que le aportara glucosa, para evitar una posible hipoglucemia, pero en estos casos no hubo problema alguno. En otros casos, ha habido algún retraso en el desarrollo psicomotor, menos agilidad que los otros niños de su edad, pero ese problema ha ido dismi-

nuyendo con el paso del tiempo, adquiriendo más resistencia y haciéndose menos notoria la diferencia respecto a los demás.

Se ha comprobado que niños que han tenido alguna debilidad muscular, dificultad de reacción ante una caída o lentitud en sus movimientos, entre otros síntomas, en los primeros años de vida, han mejorado sustancialmente a este problema en el momento que han comenzado a practicar algún deporte, adquiriendo fuerza y agilidad como cualquier otro niño, es por ello que se recomienda inculcarles ésta práctica desde muy pequeños.

DIAGNÓSTICO DE LA ENFERMEDAD

Una edad frecuente de diagnóstico de la enfermedad es alrededor de los dos años de edad, lo cual no significa que no se manifieste antes.

Suele variar la manera en cómo se diagnostica esta enfermedad. En ocasiones con una simple muestra de sangre del afectado y de la madre es suficiente para su diagnóstico, en otros casos ha sido necesario practicarles una biopsia, una punción hepática, para poder llegar a su diagnóstico, acompañados estos resultados de un estudio genético de los padres para determinar con exactitud el tipo y subtipo, y a cuál cromosoma está ligado.

Ante la sospecha de glucogenosis tipo IX, debe ponerse en marcha un proceso de diagnóstico que incluirá siempre análisis sanguíneos, así como radiografías y pruebas de ultrasonido del hígado con el objeto de detectar posibles anomalías en dicho órgano.

La glucogenosis tipo IX debe considerarse siempre dentro del diagnóstico diferencial en niños con hepatomegalia crónica aparentemente asintomática [13].

En lo referente a los análisis sanguíneos debe resaltarse que la hiperlipidemia, la hiperlactacidemia en ayuno y/o la elevación de las transaminasas sugieren el diagnóstico de deficiencia de fosforilasa kinasa, particularmente si se está ante la presencia de hepatomegalia. En los casos con afectación muscular, los niveles de CPK pueden estar por encima de lo normal.

Cuando no se cuente con un estudio genético previo, el diagnóstico de la glucogenosis tipo IX deberá incluir una medición de la actividad de la enzima Fosforilasa b Kinasa en leucocitos y/o en el tejido hepático, siempre que los síntomas, análisis sanguíneos y estudios ecográficos sugieran una posible deficiencia de dicha proteína. Es recomendable llevar a cabo una medición de la actividad de la

FBK en los leucocitos, pues dicho análisis únicamente requiere una extracción sanguínea y los resultados estarían disponibles en poco tiempo. Este tipo de estudio tiene una alta fiabilidad, aunque en algunos casos puede resultar no concluyente, por lo que para todos los pacientes resulta conveniente confirmar un diagnóstico **definitivo** de la enfermedad mediante la determinación exacta de los niveles de actividad enzimática a partir del análisis bioquímico de una biopsia hepática. En los casos con afectación muscular es conveniente llevar a cabo este mismo proceso de análisis en una biopsia del músculo esquelético.

Si existen antecedentes familiares que hayan desembocado en la realización de estudios genéticos tendentes a identificar las mutaciones de los padres, entonces es posible diagnosticar la enfermedad en nuevos afectados de una forma rápida, precisa y no invasiva, mediante una análisis de ADN, a partir de una muestra sanguínea del paciente, que confirmará la enfermedad si se advierte la presencia simultánea de las mutaciones previamente detectadas en los padres.

En cualquier caso, para todos los afectados y para sus familiares más directos siempre debe llevarse a cabo un estudio genético tendente a identificar las raíces últimas de la enfermedad. La disponibilidad en la literatura científica de un espectro cada vez más amplio de mutaciones genéticas causantes de la glucogenosis tipo IX hace que hoy en día sea cada vez más factible esta opción de confirmación del diagnóstico, que, por otra parte, permite distinguir, sin ningún género de dudas, entre los diferentes subtipos y patrones de herencia de esta patología. La identificación de las mutaciones genéticas abre también la puerta al diagnóstico prenatal de una posible futura descendencia.

SEGUIMIENTO

Es muy importante el seguimiento de la enfermedad, con una evaluación periódica dirigida por un médico preferiblemente familiarizado con enfermedades metabólicas o digestivas, para supervisar los aspectos clínicos y dar las recomendaciones dietéticas en cada caso, así como cualquier otra pauta que se deba seguir.

La periodicidad de estas evaluaciones la determina cada médico según su criterio, hay quienes las realizan una vez al año, incluso hasta cada dos años, y en otros casos hasta tres veces en un mismo año, donde normalmente se practican análisis de sangre y ecografías, y como estudios complementarios se pueden realizar otras pruebas menos comunes, como por ejemplo una densitometría ósea.

TRATAMIENTO

En lo que al tratamiento dietético se refiere, existe más diversidad entre los casos conocidos, siempre dependiendo ésta de los resultados de las analíticas, que se les suele realizar con bastante regularidad. Suelen tener un apetito normal, hay quienes tienen más y quienes tienen menos, pero hacen sus cinco tomas diarias, destacando que por las mañanas no suelen tener mucho apetito, probablemente debido a la toma nocturna de maicena.

Por lo general, si no existe ninguna contraindicación médica, la dieta a seguir es la de cualquier persona "sana", eliminando los productos que contengan azúcares directos: sacarosa (azúcar común), como golosinas, caramelos, bollería, refrescos, etc. Y, en determinados casos, limitando el consumo de fruta, debido a la fructosa que contiene, dejando ésta para tomarla en los postres (siempre después de haber ingerido suficientes carbohidratos).

Debido a la enfermedad, el colesterol podría tener tendencia a subir, por este motivo, se recomienda el consumo moderado de grasas. Por lo general es una alimentación, variada, sana y equilibrada, en la que no falte carbohidratos complejos en ninguna ingesta.

Eso sí, la toma nocturna de maicena es general para todos, puede variar la cantidad que se les dé, pero puede oscilar entre 10 y 20 gr. Unas cuatro o cinco cucharaditas de postre disueltas en leche de vaca semidesnatada, preferiblemente, procurando dárselo lo más tarde posible para que el tiempo de ayuno no sea muy prolongado y no exista riesgo de alguna bajada de glucosa, aunque hay que decir que en estos casos no es normal tener hipoglucemias.

No suelen tomar medicación alguna, pero sí en algunos casos se les han recetado algunas vitaminas, de las que su organismo carezca, en determinados períodos de tiempo, entre las conocidas que han sido recetadas a los casos que conocemos están el hierro que es para cuando están bajos de ferritina, previene la anemia; la vitamina D3 para los huesos; la vitamina A para la función de la retina.

Hasta la fecha, el tratamiento de la enfermedad se remite a terapias paliativas destinadas a minimizar la incidencia de los síntomas, principalmente a partir de unas pautas nutricionales apropiadas. El empleo de estas terapias varía significativamente de unos pacientes a otros, y no es infrecuente que encontrar a niños que evolucionan favorablemente de forma espontánea, sin recibir tratamiento alguno.

La hipoglucemia asociada a la enfermedad es, generalmente, leve y no siempre requiere tratamiento, excepto la prevención de periodos de ayuno prolongados, así como la instauración de tomas nocturnas adicionales durante episodios infecciosos. Para mantener unos niveles estables de glucosa es habitual que muchos pacientes empleen la maicena como complemento a una dieta rica en hidratos de carbono [14].

En los casos con afectación muscular se recomienda evitar el ejercicio físico intenso.

Aparte de estas medidas, no suele ser necesario imponer restricciones adicionales en los hábitos del paciente.

Debido al carácter relativamente benigno de esta patología es improbable que en un futuro inmediato surjan terapias que permitan una cura efectiva de la enfermedad, como podrían ser la terapia de substitución enzimática o las terapias génicas, pues el alto coste asociado al desarrollo y aplicación de las mismas las convierte en económicamente poco atractivas para una enfermedad rara como la glucogenosis tipo IX. Sin embargo, al ser ésta una glucogenosis relativamente común, la AEEG considera imprescindible que aumente el grado de conocimiento de la misma entre la comunidad médica, para de esta manera garantizar la generalización de un diagnóstico rápido y preciso de la enfermedad en todos los hospitales españoles, con el objeto de poner en práctica lo antes posible las pautas terapéuticas disponibles y de evitar que los pacientes sufran innecesariamente posibles secuelas como consecuencia de la falta del apropiado tratamiento de esta patología.

AGRADECIMIENTOS

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OTRAS FUENTES

- Asociación Francesa de Glucogenosis: http://www.glycogenose.org
- Asociación Alemana de Glucogenosis: http://www.glykogenose.de/
- Asociación Italiana de Glucogenosis: http://www.aig-aig.it/
- Asociación Británica de Glucogenosis: http://www.agsd.org.uk/
- Asociación Americana de Glucogenosis: http://www.agsdus.org
- Sistema de Información de Enfermedades Raras (SIRE): http://cisat.isciii.es
- Medline Plus: http://www.nlm.nih.gov/medlineplus/

Asociación Española de Enfermos de Glucogenosis (AEEG)

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Correo: amolares@gmail.com

La Asociación Española de Enfermos de Glucogenosis se encuentra integrada en:

- Federación Española de Enfermedades Raras (FEDER)
  http://www.enfermedades-raras.org
- European Organization for Rare Diseases (EURORDIS)
  http:// www.eurordis.org
- International Pompe Association (IPA)
  http:// www.worldpompe.org

GUÍA NUTRICIONAL EN GLUCOGENOSIS

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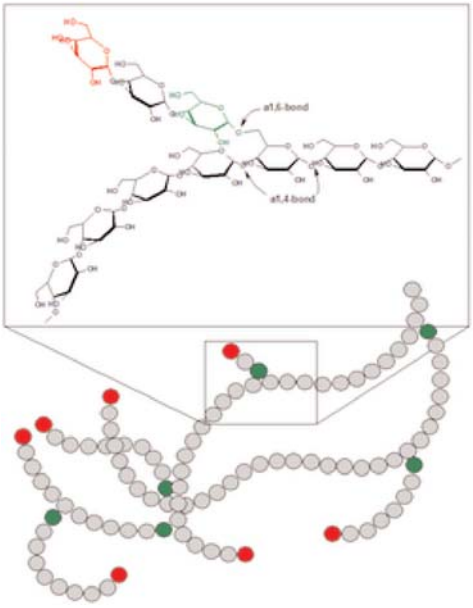
GUÍA NUTRICIONAL PARA EL PACIENTE CON GLUCOGENOSIS

La presente guía nutricional ha sido elaborada por especialistas del Servicio de Endocrinología y Nutrición del Complejo Hospitalario Universitario de Vigo, y pretende servir de ayuda a aquellos pacientes afectados de glucogenosis. Hemos querido recoger en este manual, información actualizada y útil sobre este problema. Aunque en el presente documento se recogen las pautas generales de nutrición en glucogenosis que han demostrado ser eficaces, puede ocurrir que en algunos casos concretos se necesiten algunas medidas o actuaciones especiales. En términos globales lo que se explica en esta guía es adecuado para la mayoría de los pacientes con glucogenosis, y en ningún caso va a repercutir negativamente en su salud.

Comenzaremos la guía explicando algunos apartados generales para que se comprendan mejor algunos conceptos de los que se habla posteriormente, para acabar introduciendo cada tipo de glucogenosis, recogiendo en apartados los 5 tipos principales: Ia/Ib, II, III, V y IX. Al final disponemos de un apéndice donde se explica brevemente la composición y el valor energético de los principales grupos de alimentos y un pequeño glosario de términos donde se podrán consultar algunas abreviaturas y palabras de la guía nutricional.



¿Qué es la GLUCOGENOSIS?

Un conjunto de enfermedades relacionadas con el metabolismo del glucógeno.

¿Qué es el GLUCOGENO?

Es una molécula formada por varias unidades de glucosa. Si pensamos en la glucosa como una perla, el glucógeno sería el collar.

¿Qué es la GLUCOSA?

La glucosa es un hidrato de carbono que se obtiene a partir de los alimentos y que se absorbe en el intestino pasando al torrente sanguíneo. El hígado y el músculo son capaces de almacenarlo en forma de glucógeno para que el organismo pueda tener disponibilidad de glucosa en cualquier momento.

Tipos de GLUCOGENOSIS

La glucogenosis incluye diferentes subtipos dependiendo del tipo de alteración metabólica que ocurra con el glucógeno, y se numeran desde la 0 hasta la XV, aunque de forma más sencilla podemos agruparlas en 3 tipos:

- Glucogenosis musculares (afectan principalmente a los músculos)
- Glucogenosis hepáticas (afectan principalmente al hígado)
- Glucogenosis generalizadas (con manifestaciones hepáticas, musculares y cardíacas).

Tipos de HIDRATOS DE CARBONO

La glucosa puede encontrarse en los alimentos de estas dos formas:

- Hidratos de carbono (HC) de absorción lenta o azúcares complejos: están compuestos por cadenas con muchas moléculas de glucosa (más de 8). Liberan la glucosa al torrente sanguíneo lentamente, manteniendo los valores de glucosa sanguíneos (glucemia) estables, evitando hipoglucemias.

  Fuentes alimentarias: pasta (fideos, macarrones, sopa…), arroz, cuscús, pan, trigo, avena, cebada, almidón de maíz[1], legumbres (garbanzos, alubias, lentejas…)

- Hidratos de carbono (HC) de absorción rápida o azúcares simples: están compuestos por cadenas que contienen entre 1 y 8 moléculas de glucosa. Liberan la glucosa al torrente sanguíneo muy rápido, produciendo un ascenso y una bajada muy rápida de la glucemia.

  Fuentes alimentarias: azúcar (sacarosa), leche (lactosa[2]), frutas (fructosa[2]) y miel (fructosa y glucosa[2]), otros como maltodextrinas (cereales hidrolizados[3]).

IMPORTANTE:

- Los hidratos de carbono (HC) de absorción rápida no mantienen los niveles de glucemia contantes en el tiempo, pero es imprescindible su consumo en caso de HIPOGLUCEMIA
- En la glucogenosis se recomienda el consumo preferente de alimentos con hidratos de carbono de absorción lenta.

1 Maicena

2 Estos HC al estar contenidos en alimentos que contienen proteínas, grasas o fibra, su absorción es más lenta que el azúcar (si se toma solo o con agua)

3 Ver la sección de azúcares en el apéndice “Valor energético de los principales tipos de alimentos” al final del documento.

EFECTO DE LOS AZÚCARES SIMPLES Y COMPLEJOS EN EL METABOLISMO DE LA INSULINA Y EL GLUCAGÓN

Cuando los niveles de glucosa en sangre son elevados (hiperglucemia), el organismo responde liberando una hormona, la insulina, que se encarga de reducir los niveles de glucosa en sangre; además de este efecto, la insulina es "formadora de grasa" (lipolítica), lo que significa que va a causar también un aumento de los triglicéridos y de los ácidos grasos en sangre.

Cuando los niveles de glucosa en sangre son bajos (hipoglucemia), se va a sintetizar otra hormona diferente, el glucagón, que produce el efecto contrario a la insulina. Es decir, activa varios mecanismos que aumentan los niveles de azúcar en sangre. Una de las vías que activa el glucagón es la degradación del glucógeno del hígado.

El consumo de azúcares sencillos provoca un rápido incremento en los niveles de azúcar en sangre que es corregido mediante la hormona insulina, pero como el azúcar simple se agota enseguida, ya que se absorbe muy rápido, este descenso en la glucosa sanguínea producido por la insulina hace que se tenga que activar el mecanismo compensatorio del glucagón. Sin embargo, en los pacientes con algún tipo de glucogenosis hepática, la obtención de glucosa a partir del glucógeno es nula o prácticamente nula, por lo que se producirá una hipoglucemia. Para compensar esto se pueden ingerir nuevamente azúcares simples, que provocan otra vez la subida de la glucosa sanguínea. Si esto lo prolongamos en el tiempo, veremos un efecto dientes de sierra en el perfil glucémico de un paciente con algún tipo de glucogenosis hepática, en vez de una línea constante sin apenas altibajos como ocurre en una persona sana (ver Ilustración 3).

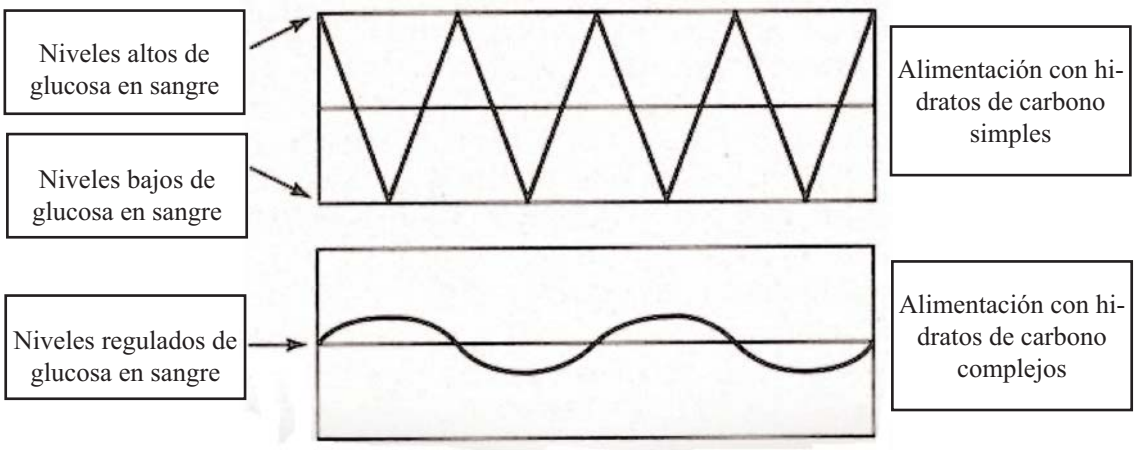


Ilustración 1

GLUCOGENOSIS TIPO Ia/Ib (ENFERMEDAD DE VON GIERKE)

Estos subtipos de glucogenosis pertenecen al grupo de las llamadas *glucogenosis hepáticas.*

Características

Existe poca o ninguna actividad de la enzima que transforma la glucosa-6-fosfato (G6P) en glucosa (enzima glucosa-6-fosfatasa o G6Pasa), o de la enzima que transporta la G6P al interior del retículo endoplasmático (enzima translocasa de glucosa-6-fosfato o TG6P).

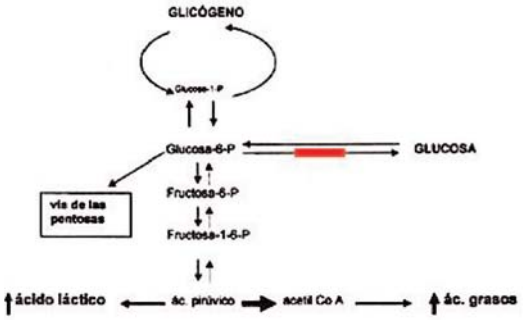


Ilustración 2

En ambos casos se interrumpe la síntesis de glucosa y la obtención de glucosa por degradación del glucógeno (ver Ilustración 2). Debido a esto la producción de glucosa hepática a partir del glucógeno no tiene lugar o es insuficiente, por lo que toda la glucosa-6-fosfato que se obtiene en la degradación del glucógeno se redirige a otras vías metabólicas (ruta de las pentosas fosfato o síntesis de ácido pirúvico/piruvato) (ver Ilustración 3).

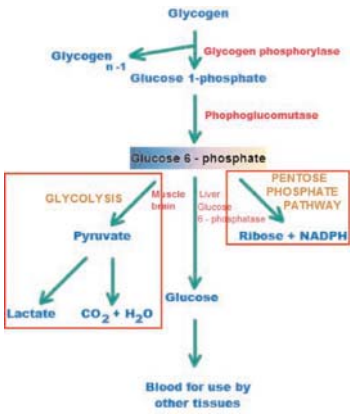


Ilustración 3

Por este motivo los pacientes tienen incrementados los niveles de ácido láctico/lactato y ácidos grasos en sangre, así como disminuidos los niveles de glucosa en sangre.

Se deben evitar los azúcares simples para evitar los picos de glucosa tan comunes en estos pacientes (ver apartado "EFECTO DE LOS AZÚCARES SIMPLES Y COMPLEJOS EN EL METABOLISMO DE LA INSULINA Y EL GLUCAGÓN"). Además ese estrés metabólico también conduce a un aumento del ácido láctico y de los triglicéridos en sangre.

Planificación alimentaria

Objetivos:

1- Mantener los niveles de glucemia en sangre dentro de la normalidad (niños: 80-130 mg/dL // adultos: 60-110 mg/dL).

2- Evitar el aumento de ácido láctico y ácidos grasos.

Composición de la dieta:

- Hidratos de carbono de absorción lenta (deben aportar entre el 60-70% de la energía diaria)
  Fuentes alimentarias: pasta (fideos, macarrones, sopa…), arroz, cuscús, pan, trigo, avena, cebada, almidón de maíz[4], legumbres (garbanzos, alubias, lentejas…)
- Grasas (deben aportar entre el 20-25% de la energía diaria)
  Fuentes alimentarias: consumo preferente aceite de oliva y pescado azul.
- Proteínas (deben aportar entre el 10-15% de la energía diaria)
  Fuentes alimentarias: carnes, pescados, huevos y lácteos (queso, yogur, leche sin lactosa).

Normas alimentarias:

- Control de glucemias constantes y prevención de hipoglucemias. Realizar comidas frecuentes, cada 2-4 horas (según tolerancia, actividad física…), ricas en HC de absorción lenta durante el día.

- **Restringir la ingesta de lactosa.** Los quesos que han sufrido un proceso de fermentación largo (quesos semicurados o curados, por ejemplo) son productos sin lactosa, ya que ésta se consume en el proceso de fermentación y maduración, por lo que se pueden consumir. Evitar los yogures, en caso de no tolerarlos, y se recomienda además no consumir leche sin lactosa, ya que las que hay disponibles en el mercado, en el proceso de elaboración, sufren un proceso de descomposición de la lactosa y contienen los productos de su hidrólisis, que son la glucosa y la galactosa. Ambos son azúcares simples que se deben evitar. Sí es muy recomendable la ingesta de leche de arroz, que carece de azúcares simples en su composición. Comparada con la leche de vaca, la leche de arroz contiene más carbohidratos, pero no contiene cantidades significativas de calcio ni de proteína. No contiene lactosa ni colesterol. La leche de arroz comercial se suele complementar con vitaminas y minerales (calcio, vitamina B_{12}, vitamina B_3, y hierro).

- **Restringir los HC de absorción rápida** (galactosa, fructosa y sacarosa) evitando aquellos alimentos o bebidas edulcoradas (especialmente si se usa el edulcorante sorbitol que se transforma en fructosa durante el proceso de la digestión). Evitar en la medida de lo posible los dulces y los productos de repostería, así como limitar la ingesta de frutas y vegetales. Sobre todo las frutas por su alto contenido en HC de absorción rápida (y fructosa que tampoco es tolerada en muchas ocasiones). La fruta más madura tiene menores proporciones de fructosa y mayores proporciones de glucosa que la verde.

- **La ingesta de almidón de maíz crudo** (AMC) antes de acostarse puede prevenir la aparición de hipoglucemias nocturnas, ya que es un HC de absorción lenta. La cantidad media suele ser de 2g/Kg para niños y de 4g/Kg para adultos.

- **Especial atención a la correcta administración de vitaminas y minerales**. Son frecuentes dietas carenciales en vitaminas y minerales al limitar la ingesta de frutas,

4 Maicena

verduras y lácteos. Para ello, se puede recurrir a los suplementos de vitaminas y minerales, pero siempre bajo supervisión médica, ya que las vitaminas liposolubles (A, D, E y K) pueden generar complicaciones si se toman en exceso. Debemos prestar especial importancia a los siguientes:

- Hierro: Se debe tener especial precaución con la suplementación del mismo, ya que un exceso del aporte de hierro puede favorecer la aparición de adenomas. Los adenomas hepáticos son tumores benignos, que pueden tener un riesgo de rotura y por tanto de hemorragia, lo que puede llevar a un cuadro de shock con hipotensión, taquicardia y sudoración, e incluso progresar a una dolencia maligna.

- Calcio: Es importante conseguir un buen aporte dietético diario (soja, leche de soja y de arroz, cereales enriquecidos, quesos curados, espinacas, brócoli, alubias blancas, almendras, higos secos…) para evitar la pérdida de masa ósea. Siempre se aconseja vigilar los niveles de calcio por un especialista, ya que el calcio tomado en exceso puede formar cristales de oxalato cálcico y derivar en una litiasis renal (piedras en el riñón). La vitamina D estimula la absorción de calcio, mientras que el hierro dificulta su absorción en el intestino.

- Selenio y Zinc: Estos 2 minerales participan en procesos como el de la formación de enzimas antioxidantes y ayudan a estimular la respuesta inmunitaria. Además el zinc es un protector hepático, por lo que es recomendable ingerir las cantidades diarias adecuadas para cubrir las necesidades normales del organismo.

- Vitamina C: Aparte de se un antioxidante natural, la vitamina C participa en procesos como la cicatrización de heridas y la reparación y mantenimiento del tejido óseo.

- Vitamina D: Su importancia reside en que estimula la absorción de calcio a nivel intestinal, por lo que debido a los problemas de osteopenia que sufren algunos pacientes de glucogenosis tipo I es recomendable vigilar sus niveles.

• **En los pacientes con glucogenosis tipo Ib se puede valorar la suplementación con vitamina E**, ya que aunque no está demostrada su utilidad, se ha visto que puede ser de ayuda en algunos pacientes. Juega un rol importante en el sistema inmune y los procesos metabólicos del cuerpo. Fuentes naturales de vitamina E son los frutos secos, semillas, el aceite de oliva, la margarina y otros aceites vegetales. Siempre bajo supervisión médica, ya que los suplementos de vitamina E pueden ser dañinos para las personas que toman anticoagulantes.

• **Deben evitarse:**

- El consumo de esteroides. Dichas sustancias pueden producir desde deterioro en la función excretora del hígado, hasta fallo hepático e incluso tumores, por lo que debe evitarse su consumo excepto por prescripción médica y siempre baja una estricta supervisión.

- El consumo de alcohol. Por un lado se evitan las hipoglucemias causadas por la ingesta del mismo, y por otro lado se evita un empeoramiento de la hepatopatía causada por la propia glucogenosis.

GLUCOGENOSIS TIPO II (ENFERMEDAD DE POMPE)

La glucogenosis tipo II pertenece al tipo de las glucogenosis generalizadas.

Características

Se produce una acumulación creciente de glucógeno dentro de un compartimento celular llamado lisosoma (ver Ilustración 4). Esta acumulación se debe a que hay una ausencia total o parcial en el lisosoma de la enzima encargada de descomponer el glucógeno en moléculas de glucosa (α-(1-4)-glucosidasa o también llamada maltasa ácida). Esta deficiencia puede tener consecuencias sobre diferentes tejidos, aunque el efecto más notable se produce en las células musculares, pues en ellas se acumula gran cantidad de glucógeno residual que es absorbido por los lisosomas para su transformación en glucosa.

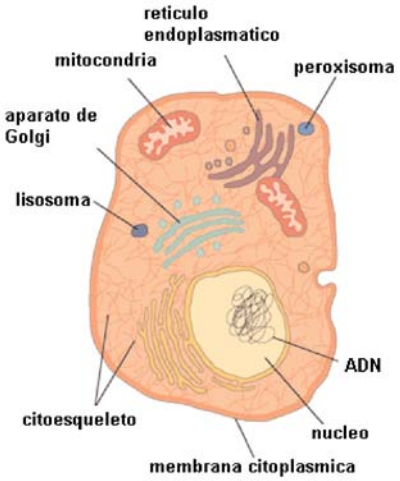


Ilustración 4

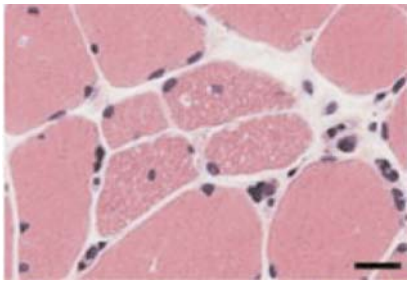


Ilustración 5

El depósito creciente de glucógeno en los lisosomas interfiere con la función celular y causa daños en las células (ver Ilustración 5). En niños destaca por producir insuficiencia cardíaca al acumularse en el músculo cardíaco, causando cardiomegalia.

Planificación alimentaria

Objetivos:

Mantener los valores de glucosa en sangre dentro de la normalidad y evitar en la medida de lo posible la necesidad de que el organismo tenga que recurrir a la degradación de glucógeno a través de la vía lisosomal. Una dieta baja en HC ayudará a que los depósitos de glucógeno en el tejido sean menores. Asimismo se incrementarán las proporciones de otros nutrientes de los que podemos obtener energía como las proteínas o las grasas.

Composición de la dieta:

- Hidratos de carbono de absorción lenta (deben aportar aproximadamente el 50% de la energía diaria)

Fuentes alimentarias: pasta (fideos, macarrones, sopa…), arroz, cuscús, pan, trigo, avena, cebada, almidón de maíz[5], legumbres (garbanzos, alubias, lentejas…)

- Grasa (deben aportar entre el 20-25% de la energía diaria)
  Fuentes alimentarias: consumo preferente de aceite de oliva y pescado azul.
- Proteínas (deben aportar entre el 20-25% de la energía diaria)
  Fuentes alimentarias: carnes, pescados, huevos y lácteos (queso, yogur, leche sin lactosa)

Normas alimentarias:

• **Especial atención a la correcta administración de vitaminas y minerales:** Son frecuentes dietas carenciales en vitaminas y minerales al limitar la ingesta de frutas, verduras y lácteos. Para ello, se puede recurrir a los suplementos de vitaminas y minerales, pero siempre bajo supervisión médica, ya que las vitaminas liposolubles (A, D, E y K) pueden generar complicaciones si se toman en exceso.

• En algunos pacientes con glucogenosis tipo II **puede ser beneficioso un aporte extra de alanina** en forma de suplemento, y siempre bajo supervisión médica. No ha demostrado su beneficio, pero la alanina es un precursor de glucosa y puede ayudar a prevenir el agotamiento muscular cuando el glucógeno se acumula en las células.

• Algunos pacientes que sufren de extrema debilidad pueden llegar a requerir de **alimentación mediante sonda** para mejorar su estado nutricional. En estos casos siempre bajo supervisión y recomendación del médico especialista correspondiente.

• **Trocear los alimentos:** Para comer más fácilmente, los alimentos pueden mezclarse, cortarse en partes pequeñas o tomarse junto con algún tipo de salsa o crema añadidas. Es recomendable para facilitar la ingestión y digestión tomar bocados pequeños y masticar muy bien la comida.

• **Hidratarse correctamente:** Asegurarse de beber suficientes líquidos a lo largo del día, tomándolos lentamente. El uso de pajitas y el mantenerse derecho al menos durante 1-2h tras la comida puede resultar útil.

• **Uso de espesantes en casos de problemas en la deglución:** Algunos alimentos pueden pasar muy deprisa por la garganta, por lo que si existen problemas de atragantamiento con determinadas comidas (sopas, caldos, agua o leche) es recomendable la utilización de espesantes como la crema de arroz infantil, la maicena, o los espesantes artificiales de farmacia.

• **Deben evitarse:**

- El consumo de esteroides. Dichas sustancias pueden producir desde deterioro en la función excretora del hígado, hasta fallo hepático e incluso tumores, por lo que debe evitarse su consumo a no ser que sea por prescripción médica y baja una estricta supervisión.
- El consumo de alcohol. Por un lado se evitan las hipoglucemias causadas por la ingesta del mismo, y por otro lado se evita un empeoramiento de la hepatopatía causada por la propia glucogenosis.

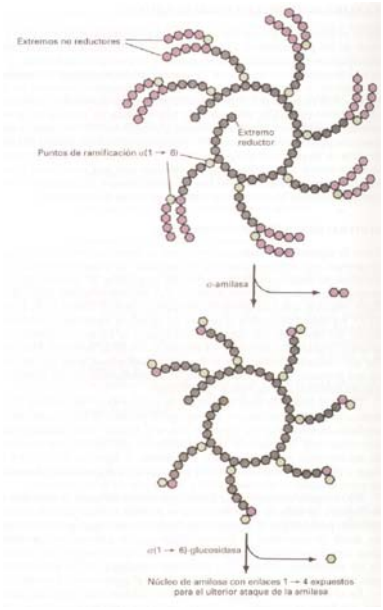
5 Maicena

GLUCOGENOSIS TIPO III (ENFERMEDAD DE CORI-FORBES)

La glucogenosis tipo III pertenece al grupo de las glucogenosis hepáticas.

Características

Se produce una acumulación de cadenas cortas de glucógeno en el interior de las células. Esta degradación incompleta y anormal del glucógeno, se debe a que la enzima responsable del proceso de ruptura de las moléculas de glucosa en los puntos de ramificación (amilo-1,6-glucosidasa – A1,6G), o bien está ausente o bien tiene muy baja actividad. Así, estos pacientes presentan una sintomatología parecida a un enfermo de glucogenosis tipo I, pero de una forma más leve. La mayoría de los enfermos de glucogenosis tipo III tienen deficiencia de A1,6G tanto en el hígado como en el músculo (subtipo IIIa), pero cerca del 15% tienen deficiencia de la enzima únicamente en el hígado (subtipo IIIb).



Planificación alimentaria

Objetivos:

1- Mantener los niveles de glucemia en sangre dentro de la normalidad (niños: 80-130 mg/dL // adultos: 60-110 mg/dL).

2- Evitar el aumento de ácido láctico y ácidos grasos.

En este tipo de glucogenosis la degradación del glucógeno hasta los puntos de ramificación da lugar a moléculas de glucosa, por lo que las hipoglucemias y el aumento de ácidos grasos y de ácido láctico son menores.

Composición de la dieta:

- Hidratos de carbono de absorción lenta (deben aportar aproximadamente el 50% de la energía diaria)

  Fuentes alimentarias: pasta (fideos, macarrones, sopa…), arroz, cuscús, pan, trigo, avena, cebada, almidón de maíz[6], legumbres (garbanzos, alubias, lentejas…)

- Grasas (deben aportar el 25% de la energía diaria)

  Fuentes alimentarias: consumo preferente aceite de oliva y pescado azul.

- Proteínas (deben aportar el 25% de la energía diaria)

  Fuentes alimentarias: carnes, pescados, huevos y lácteos (queso, yogur, leche sin lactosa).

Normas alimentarias:

- **Evitar ayunos prolongados.** Para ello se deben realizar comidas frecuentes ricas en HC de absorción lenta durante el día. Es recomendable realizar ingestas cada 4-6h. Con ello se intentan prevenir las hipoglucemias y mantener los niveles de glucosa constantes a lo largo del día.

6 Maicena

• **Restringir los HC de absorción rápida** (galactosa, fructosa y sacarosa) evitando aquellos alimentos o bebidas edulcoradas (especialmente si se usa el edulcorante sorbitol que se transforma en fructosa durante el proceso de la digestión). Evitar en la medida de lo posible los dulces y los productos de repostería, así como limitar la ingesta de frutas y vegetales. Sobre todo las frutas por su alto contenido en HC de absorción rápida (y fructosa que tampoco es tolerada en algunas situaciones). La fruta más madura tiene menores proporciones de fructosa y mayores proporciones de glucosa que la verde.

• En un estudio se ha descrito que en aquellos pacientes con glucogenosis tipo III y cardiomiopatía, **puede resultar beneficioso la suplementación con 3-Hidroximetilbutirato** (HMB) además de la dieta rica en proteína. El HMB se encuentra como suplemento en polvo y puede actuar como agente anticatabólico, reducir las lesiones en las fibras musculares debidas al ejercicio, incrementar la fuerza, e incluso juega un papel en la función inmunológica.

• **En lactantes y niños:**

- Realización de comidas pequeñas y frecuentes.
- Introducción del almidón de maíz crudo en el primer año de vida si la hipoglucemia está presente.
- El aporte de HC complejos, almidón de maíz crudo, y/o alimentación enteral continua, puede evitar hipoglucemias durante la noche. En el caso de la ingesta de almidón de maíz crudo (AMC) antes de acostarse la dosis recomendada suele ser de 1,6g/Kg cada 4 horas.

• **En adolescentes y adultos:**

- En el caso de personas con miopatías, puede ser beneficiosa la ingesta de leche semidesnatada enriquecida con proteínas o de suplementos con fórmulas hiperproteicas antes de acostarse.
- En la glucogenosis tipo IIIb, se irán cambiando las restricciones dietéticas a una dieta normal bien balanceada.
- La ingesta de grasas poliinsaturadas pueden ser de utilidad para reducir la hipercolesterolemia.
- El aporte de HC complejos, almidón de maíz crudo, y/o alimentación enteral continua, puede evitar hipoglucemias durante la noche. La cantidad media recomendada de almidón de maíz crudo antes de acostarse suele ser de 1g/Kg cada 4 horas en aquellos adultos con una sintomatología leve, y de entre 1,7-2,5g/Kg cada 4 horas en los casos más graves.

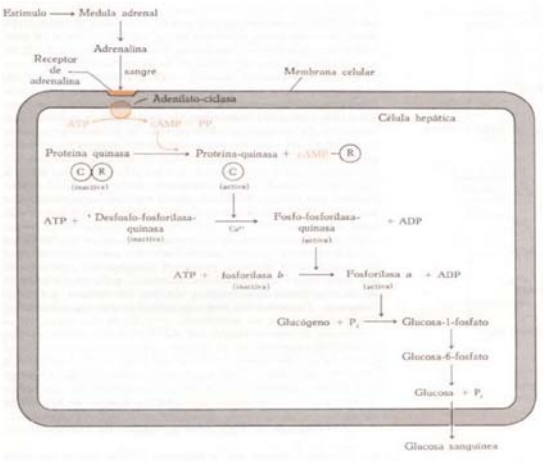
• **Deben evitarse:**

- El consumo de esteroides. Dichas sustancias pueden producir desde deterioro en la función excretora del hígado, hasta fallo hepático e incluso tumores, por lo que debe evitarse su consumo a no ser que sea por prescripción médica y baja una estricta supervisión.

- El consumo de alcohol. Por un lado se evitan las hipoglucemias causadas por la ingesta del mismo, y por otro lado se evita un empeoramiento de la hepatopatía causada por la propia glucogenosis.

GLUCOGENOSIS TIPO V (ENFERMEDAD DE McARDLE)

La glucogenosis tipo V o también llamada enfermedad de McArdle, pertenece al grupo de las *glucogenosis musculares.*



Características

Existe un déficit o una baja actividad de la enzima fosforilasa del músculo, siendo normal la actividad de dicha enzima en hígado. Dado que el glucógeno del hígado se descompone para poder suministrar glucosa al resto del organismo, no se producen hipoglucemias, pero existe un aumento de glucógeno leve en músculo. Este glucógeno muscular es usado por las células contráctiles del músculo como energía. Al no poder descomponerse este glucógeno, lo que ocurre es una mala tolerancia al ejercicio desde la infancia y se caracteriza por debilidad y calambres temporales del músculo esquelético durante el mismo.

Planificación alimentaria

Objetivos:

Lo más normal es observar un tratamiento sintomático, evitando la realización de ejercicio físico intenso. Asimismo se deben evitar las dietas excesivamente bajas en carbohidratos.

Composición de la dieta:

- Hidratos de carbono de absorción lenta (deben aportar aproximadamente el 40% de la energía diaria)

  Fuentes alimentarias: pasta (fideos, macarrones, sopa…), arroz, cuscús, pan, trigo, avena, cebada, almidón de maíz[7], legumbres (garbanzos, alubias, lentejas…)

- Grasas (deben aportar entre el 25-30% de la energía diaria)

  Fuentes alimentarias: consumo preferente aceite de oliva y pescado azul.

- Proteínas (deben aportar entre el 25-30% de la energía diaria)

  Fuentes alimentarias: carnes, pescados, huevos y lácteos (queso, yogur, leche sin lactosa).

Normas alimentarias:

Lo que vamos a comentar a continuación no va a solucionar ni a servir de tratamiento para la glucogenosis tipo V, pero sí que podría disminuir alguno de sus efectos secundarios asociados a la realización de ejercicio. Obviamente también hay que tener en cuenta que la actividad enzimática va a ser importante a la hora de determinar la capacidad para hacer ejercicio del paciente.

7 Maicena

• **Suplementación con BCAA's**[8]**, vitamina B_6**[9] (frutos secos, plátanos, hígado, leguminosas), creatina (carnes, pescados como el arenque y el salmón, lácteos y huevos) y/o **vitamina E** (frutos secos, aceite de oliva). Durante el ejercicio, el músculo al no poder obtener energía de la degradación del glucógeno, demanda glucosa del hígado, y además empieza a descomponer las proteínas para obtener energía. Los BCAA's propocionan energía a los músculos y detienen esa degradación de proteínas, favoreciendo el mantenimiento de la masa muscular. Los aminoácidos de cadena ramificada se encuentran en algunos suplementos para deportistas. En cuanto a la vitamina B_6, incrementa el rendimiento muscular y la producción de energía, ya que favorece la degradación del glucógeno para obtener glucosa tanto en el hígado como en el músculo. Si bien en el músculo no va a ayudar, puesto que falta la enzima responsable, si puede ayudar a que la descomposición del glucógeno hepático sea más eficiente. Además puede facilitar la pérdida de peso porque ayuda a que el organismo consiga energía a partir de las grasas acumuladas. La creatina ayuda a incrementar los depósitos de energía en el músculo, con lo que ayuda a disminuir un poco la fatiga. Aparte de glucosa, los músculos necesitan del ATP para poder contraerse, y la creatina se almacena en forma de fosfocreatina, generando ATP para la contracción muscular. Además la creatina favorece el aumento de la masa muscular, por lo que contribuye a tener unos músculos sanos y fuertes. Por último, se ha demostrado que la vitamina E mejora la capacidad de transporte de la creatina a los músculos. El médico es el que debe supervisar y aconsejar siempre si se deben tomar o no estos suplementos. Nunca tomarlos por cuenta propia.

• **Consumo de bebidas isotónicas con sacarosa** (glucosa + fructosa) **antes de ejercicio programado.** Se ha comprobado que la administración de sacarosa (azúcar común), en dosis de 37g en adolescentes y adultos y entre 18-20g en niños justo antes de la realización de un ejercicio se ve reflejada en una menor sensación de agotamiento.

• **El Hidroximetilbutirato (HMB)** puede actuar como agente anticatabólico, reducir las lesiones en las fibras musculares debidas al ejercicio, incrementar la fuerza, e incluso juega un papel en la función inmunológica, por lo que puede resultar beneficiosa la suplementación con este precursor de la leucina en algunos casos de debilidad muscular. El HMB se encuentra como suplemento en polvo y se debe siempre consultar al médico y no tomarlo sin su supervisión.

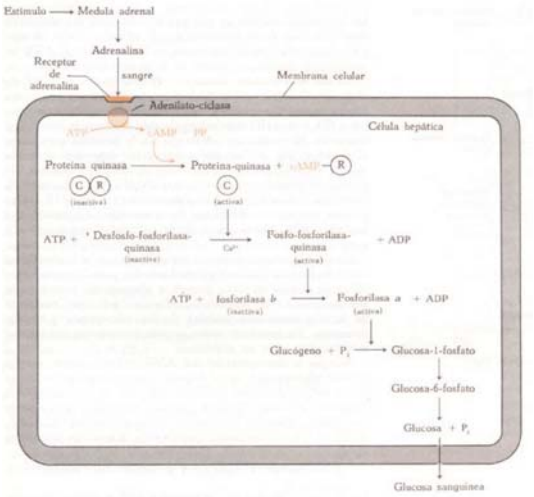
• **Valorar suplementación con vitamina B_{12}**[10]**, biotina**[11]**, vitamina D, alanina, carnitina y Co-Q_{10}**[12]**.** No se ha demostrado en ensayos clínicos su eficacia, pero puede ser de utilidad, al tener un papel en diferentes mecanismos de contracción y nutrición muscular. Siempre se debe acudir al especialista para tratar todo tipo de suplementaciones, es decir, siempre bajo supervisión médica.

• **Debe evitarse el consumo de alcohol.** En este tipo de glucogenosis no se trata de que pueda causar hipoglucemias o que empeoren la hepatopatía, pero si que debemos respetar y cuidar el hígado para que tenga un funcionamiento correcto, ya que es el suministrador de glucosa del organismo y del músculo, al ser éste incapaz de aprovechar el glucógeno que tiene almacenado.

8 Aminoácidos de cadena ramificada (valina, leucina e isoleucina)
9 Piridoxina
10 Cobalamina
11 Vitamina B_8 o vitamina H
12 Coenzima Q_{10}

GLUCOGENOSIS TIPO IX

La glucogenosis tipo IX pertenece al grupo de las *glucogenosis hepáticas.* Tiene la particularidad de que a diferencia del resto de glucogenosis hepática, ésta está ligada al sexo, en concreto al cromosoma X.



Características

La glucogenosis tipo IX se parece desde el punto de vista bioquímico a la glucogenosis tipo II, ya que afecta a una de las fosforilasas. En este caso la enzima deficiente es la Fosforilasa b Kinasa (FBK). Esta enzima se encarga de activar a la fosforilasa a, que es una de las enzimas implicadas en la degradación del glucógeno. La ausencia o deficiencia de la FBK provoca la inactividad de la fosforilasa a, lo que se traduce en una acumulación de glucógeno en el hígado y en el músculo.

La glucogenosis tipo IX es una de las formas más benignas de glucogenosis, ya que provoca síntomas durante la infancia y adolescencia. Con la edad, las secuelas clínicas y bioquímicas propias de la enfermedad tienden a remitir gradualmente, y la mayor parte de los adultos son asintomáticos.

Planificación alimentaria

Objetivos:

1. Los objetivos son evitar las hipoglucemias.
2. Mantener unos valores reducidos de triglicéridos y de colesterol en sangre.

Composición de la dieta:

- Hidratos de carbono de absorción lenta (deben aportar entre el 60-70% de la energía diaria)

  Fuentes alimentarias: pasta (*fideos, macarrones, sopa…*), arroz, cuscús, pan, trigo, avena, cebada, almidón de maíz[13], legumbres (*garbanzos, alubias, lentejas…*)

- Grasas (deben aportar el 15% de la energía diaria)

  Fuentes alimentarias: consumo preferente aceite de oliva y pescado azul.

- Proteínas (deben aportar entre el 15-25% de la energía diaria)

  Fuentes alimentarias: carnes, pescados, huevos y lácteos (*queso, yogur, leche sin lactosa*).

Normas alimentarias:

- Antes de acostarse puede ser beneficioso **realizar una ingesta de comida ligera, hiperproteica** (carnes, pescados, huevos y lácteos sin lactosa) **y con almidón de maíz.** Con ello mantendremos unos valores de glucosa en sangre más o menos constantes durante toda la noche y proveeremos de un sustrato proteico para la obtención de energía.

13 Maicena

• **Uso de grasas poliinsaturadas**: leche, pescado azul, nueces, aceite de soja… Al tener unos niveles de colesterol elevados, los enfermos de glucogenosis se ven favorecidos por la presencia de ácidos grasos omega-3 y omega-6 en los alimentos antes mencionados. Estos ácidos grasos poliinsaturados (ácido linoleico y ácido linolénico) son esenciales para el organismo, y son cardiosaludables, protegiendo nuestro corazón y ayudando a disminuir los niveles de colesterol en sangre.

• **Productos que contengan esteroles o estanoles vegetales**, como los presentes en ciertos yogures bebibles o leches enriquecidas, también pueden contribuir a disminuir los niveles de colesterol en sangre. Diversos estudios científicos han demostrado que la ingesta diaria de 2g de esteroles/estanoles vegetales disminuyen dichos niveles.

• Evitar ayunos prolongados.

• **Deben evitarse**:

- **Los ayunos prolongados**. Para ello deben realizarse comidas frecuentes ricas en HC de absorción lenta durante el día. Es recomendable realizar ingestas cada 6h. Con ello se intentan prevenir las hipoglucemias y mantener los niveles de glucosa constantes a lo largo del día. Podemos recurrir al almidón de maíz en dosis de 0,6-2,5g/Kg cada 6 horas según requerimientos de cada uno.

- **El consumo de esteroides**. Dichas sustancias pueden producir desde deterioro en la función excretora del hígado, hasta fallo hepático e incluso tumores, por lo que debe evitarse su consumo a no ser que sea por prescripción médica y baja una estricta supervisión.

- **El consumo de alcohol**. Por un lado se evitan las hipoglucemias causadas por la ingesta del mismo, y por otro lado se evita un empeoramiento de la hepatopatía causada por la propia glucogenosis.

VALOR ENERGÉTICO DE LOS PRINCIPALES TIPOS DE ALIMENTOS:

• Leche y derivados lácteos → Poseen un alto contenido en proteínas (en torno a un 80-90%). Alrededor de un 50% del valor energético de la leche proviene de las grasas (3,6 g de grasa/100ml), con un alto contenido en grasas saturadas, por lo que se debe tener en cuenta en personas con problemas cardiovasculares y obesos. Se deben evitar las leches y los yogures con lactosa, no presentando problemas los quesos al no contener lactosa. Fuente muy importante de calcio y de fósforo, y también contiene yodo, selenio y cromo. Entre las vitaminas más destacables se encuentran la vitamina B_{12}, la riboflavina[14], vitamina A (siempre que la leche sea semidesnatada o entera), la niacina[15] y la piridoxina[16].

14 Vitamina B_2
15 Vitamina B_3
16 Vitamina B_6

• Carnes → Contenido proteico en torno al 16-25%, de alto valor biológico y con un 40% de sus aminoácidos que son esenciales en la dieta humana. Contenido en grasa variable, oscilando entre el 5-10% de las carnes magras y el 10-30% de las carnes grasas. Contenido importante en selenio, zinc, cobre, magnesio, cobalto, fósforo, cromo, níquel y sobre todo hierro. En cuanto a vitaminas, cantidades importantes de vitaminas B_3 y B_{12} y más moderadas de vitaminas B_1 y B_6.

• Embutidos → Contenido similar a las carnes, teniendo menos contenido en vitamina B_9[17] y B_6. Aportan los beneficios de los ingredientes adicionales como el ajo (bueno para la hipertensión, el reumatismo, la flatulencia, y el colesterol), la cebolla (diurética, estimulante, digestiva, correctora del estreñimiento), la pimienta o las hierbas aromáticas como el anís o el tomillo (ayudan a digerir las grasas de los alimentos cárnicos).

• Pescados → Sus principales componentes son el agua (entre un 60-80%), las proteínas (15-27% y de alto valor biológico y alta digestibilidad) y los lípidos, dependiendo de la especie:

- Azul o graso (6-25% de grasa): Anguila, atún, bonito del norte, boquerón, caballa, estornino, jurel, palometa negra o japuta, salmón, sardina…
- Semigraso (2,5-6% de grasa): Dorada, lubina, pez espada, salmonete, trucha…
- Blanco o magro (hasta el 2,5% de grasa): Acedía, bacaladilla, besugo, gallo, lenguado, merluza, rape, rodaballo…

El contenido en lípidos del pescado es generalmente Omega-3 (ácido eicosapentaenoico - EPA y ácido docosahexaenoico o DHA) y también una fuente de ácidos grasos esenciales como los ácidos grasos linoleico y linolénico.
Son ricos en minerales como calcio, fósforo, hierro, flúor, magnesio, zinc y cobre, y si son de agua salada, ricos en yodo. Son generalmente bajos en sodio y una buena fuente de potasio. En cuanto a las vitaminas, son ricos en vitaminas del grupo B (B_1[18], B_2[19], B_3[20] y B_{12}), y en caso de pescados grasos, ricos en vitaminas A y D.

• Mariscos → Bajo contenido en grasas poliinsaturadas (entre 0,5-2,5%), y alto contenido en proteínas (14-20%) y oligoelementos como minerales y vitaminas. Tienen más contenido en calcio que el pescado, y también contienen fósforo, yodo, potasio, hierro, flúor, zinc, cobre y magnesio. Rico en vitaminas B_1 y B_3. Su contenido calórico es de unas 100Kcal/100g. Precaución en individuos con alto contenido en ácido úrico.

• Huevos → Gran contenido proteico, siendo la proteína de más alto valor biológico al contener todos los aminoácidos esenciales. La yema es rica en colesterol. En cuanto a vitaminas, el huevo destaca por su riqueza en vitamina A, B_9, B_8, vitamina D. Tiene un contenido importante en colina (su ingesta deficiente produce alteraciones hepáticas, pancreáticas, renales y del crecimiento). Es un alimento de fácil digestión (sobre todo pasado por agua), y las proteínas, minerales y vitaminas que contienen se absorben en su mayor parte. La grasa de la yema se absorbe fácilmente al estar emulsionada y la presencia de fosfolípidos también favorece su absorción.

17 Ácido fólico
18 Tiamina
19 Riboflavina
20 Niacina

• Cereales → Los principales cereales utilizados son el arroz, el trigo, el maíz, la avena, la cebada, el centeno, el mijo, el sorgo y el triticale. Contienen unos 70-78% de HC, un 6-13% de proteína y un 1-7% de grasa. El almidón es el constituyente digerible mayoritario. Contienen todos los aminoácidos esenciales, aunque son deficitarios en lisina. Se aprovecha entre un 53-62% del contenido proteico de los cereales. Entre las grasas destacan las insaturadas, siendo el ácido graso linoleico el principal. Son ricos en vitaminas del grupo B (B_1, B_2 y B_3 principalmente), y en minerales como el fósforo y el potasio, aunque también contienen calcio, hierro y magnesio.

• Verduras → Su principal valor nutritivo deriva de su contenido en minerales, vitaminas y fibra. En general, son ricas sobre todo en potasio, seguido del calcio, el sodio y el magnesio y en cuanto a vitaminas, destacan la A, la C, la E, la K y del grupo B. Al formar un grupo tan heterogéneo, se recomienda variar el tipo de verduras de la dieta.

• Legumbres → Son una fuente excepcional de proteínas (entre el 20-30%), fibra (entre 10-20%), HC de digestión lenta (almidón presente con un contenido entre el 75-80%) y vitaminas del grupo B. Las proteínas de las legumbres no tienen el valor biológico de las de la leche, huevo, carne o pescado, por lo que debe alternarse su consumo con estos otros alimentos. Entre los minerales destaca de forma general el calcio, el hierro y el fósforo, pero su absorción es baja en comparación con la de otros alimentos.

• Frutas → Su composición es muy variada, dependiendo de la familia a la que pertenezcan. Su contenido en agua oscila entre un 81-93%. Presentan un alto contenido en HC (principalmente sacarosa, glucosa y fructosa), fibra, vitaminas (principalmente de los grupos A y C) y minerales (los más predominantes son el potasio y el fósforo, y bajo contenido en sodio). Debido a su alto contenido en HC de absorción rápida, se recomienda reducir su consumo en ciertos tipos de glucogenosis. Su contenido en proteínas y grasas es prácticamente nulo.

• Frutos secos → Importante fuente de grasas monoinsaturadas, excepto las nueces, que son ricas en ácidos grasos poliinsaturados (omega-6 y omega-3). Son también ricos en arginina, fibra, minerales (calcio, magnesio, fósforo y potasio entre otros) y vitamina E. Se han propuesto varios efectos cardiosaludables, y su consumo se asocia con menor riesgo de enfermedad coronaria, muerte súbita o desarrollo de obesidad o diabetes tipo II.

• Azúcares → Como azúcares nos referimos a los simples o monosacáridos, como la fructosa, la glucosa o la galactosa y a la combinación de estos, los disacáridos: sacarosa o azúcar de mesa (glucosa + fructosa), maltosa (glucosa + glucosa) y lactosa o azúcar de la leche (galactosa + glucosa). A mayores de estos también se incluyen los fructooligosacáridos (2 o 3 moléculas de fructosa + sacarosa), que se añaden a los alimentos como prebióticos para estimular el crecimiento de las bifidobacterias, las maltodextrinas, que están formadas por varias unidades de glucosa (entre 5 y 18 normalmente) y los polialcoholes, que se utilizan como edulcorantes (sorbitol, xilitol y manitol, principalmente). Alimentos como la miel, o los productos de confitería suelen ser ricos en este tipo de azúcares, en torno a un 60-99% de su composición.

• Grasas y aceites → Diferenciamos 3 tipos de grasas:

- De origen animal (mantecas y sebos): Ricas en ácidos grasos saturados y en el

caso de la grasa de cerdo, ricas en ácido oleico. La mantequilla se hace más digestible por la presencia de ácidos grasos de bajo peso molecular.

- De origen marino: Aportan fundamentalmente ácidos grasos poliinsaturados (omega-3). Son beneficiosos para el organismo, pero deben consumirse de forma moderada y acompañarse de antioxidantes como la vitamina E.
- De origen vegetal: Son ricas en ácidos grasos insaturados como el ácido linoleico (omega-6). Son ejemplos el aceite de maíz, el de cacahuete o el de girasol. El aceite de soja además es rico también en ácido linolénico (omega-3). Todos estos aceites son dietéticamente muy aceptables, no así la grasa de coco, que es muy aterogénica. En cuanto al aceite de oliva, es el más recomendable dietéticamente, ya que tiene un alto contenido en ácido oleico, una proporción adecuada de omega-6/omega-3 y una cantidad elevada de antioxidantes.

La industria somete a las grasas y a los aceites a procesos para hacerlas aptas para el consumo, pero con el inconveniente de que los productos elaborados contienen grasas trans, con efectos metabólicos no deseables. Este tipo de grasas son claramente identificables en el etiquetado de los productos de consumo, ya que en la etiqueta pueden aparecer como "grasas parcialmente hidrogenadas"

• Bebidas alcohólicas → Dentro de estas bebidas se incluyen aquellas con un porcentaje superior al 0,5% vol/vol, o lo que es lo mismo, con un grado alcohólico mayor a 0,5. Todas tienen etanol en su composición, y la cantidad variará dependiendo del tipo de bebida que sea. El valor energético del etanol es de 7 Kcal/g. A pesar de que estudios epidemiológicos han demostrado que un consumo de entre 10-80 g de alcohol/día está asociado a una disminución de la incidencia de muerte por enfermedad coronaria, debido a que el proceso de metabolización del alcohol ocurre en el hígado, se recomienda evitar el consumo de alcohol en la medida de lo posible, para minimizar el estrés hepático.

• Bebidas refrescantes → Incluyen en su composición agua, en cantidades superiores a un 85%, HC en forma de azúcares sencillos (monosacáridos y disacáridos), diferentes vitaminas (especialmente hidrosolubles como las del grupo B o la vitamina C) y minerales (generalmente calcio, sodio y magnesio) y en determinados casos incluso proteínas (en bajas cantidades). Suele desaconsejarse su consumo sobre todo en las glucogenosis Ia/Ib, II, III y IX.

• Café, té y cacao → Trataremos a estos productos de forma individual y a pesar de que su composición puede diferir dependiendo de la variedad, de la zona de cultivo, y del proceso de elaboración posterior, intentaremos dar unas pautas generales:

- Café: El contenido en HC de la infusión de café (150ml a una concentración de 50g/l) es de un 20-25%, con prácticamente un 0% de azúcares simples, que se pierden en el proceso de tueste. El contenido en lípidos es del 1%, y el de proteínas en torno a un 6%. En cuanto al contenido en minerales, los principales son el potasio, el calcio y el magnesio; otros como el manganeso, el rubidio y el cobre aparecen en niveles traza. Otro componente importante es la cafeína (5%), que es una sustancia estimulante.
- Té: Refiriéndonos siempre a una infusión (150ml de té negro a una concentración

de 50g/l y con un tiempo de infusión de unos 3 minutos), el té contiene un 4% de aminoácidos, un 4% de HC, y trazas de lípidos. El contenido en minerales es de un 5%, entre los que se encuentran: calcio, magnesio, cromo, manganeso, hierro, cobre, zinc, molibdeno, selenio, sodio, fósforo, cobalto, estroncio, níquel, potasio, flúor y aluminio en diferentes proporciones dependiendo de la variedad del producto. El cromo, el selenio y el zinc tienen propiedades antioxidantes.

- Cacao: Los productos solubles de cacao tienen por término medio un 78-82% de HC, con predominio de azúcares simples. El contenido en fibra está en un 7%, y el de proteínas entre un 4-7%. La grasa supone un 3% y corresponde a la manteca de cacao, en cuanto a vitaminas y minerales destaca el ácido fólico (vitamina B9) y el potasio, el magnesio y el calcio. En cuanto al chocolate, contiene entre un 47-65% en forma de HC, siendo prácticamente todos ellos azúcares simples. En torno a un 6-9% de proteínas dependiendo si es con leche o no, y a un 29-32% de grasa dependiendo de lo mismo. Como vitaminas destacan la B_1 y la B_9 y la A en caso de ser chocolate con leche, y en cuanto a minerales tenemos el potasio como mayoritario, el magnesio y el fósforo. Si es con leche además aportamos calcio y otros elementos como el selenio y el fósforo.

GLOSARIO:

- BCAA's → Brain chain aminoacids (en castellano aminoácidos de cadena ramificada). Son la valina, la leucina y la isoleucina.
- G6P → Glucosa 6 fosfato.
- G6Pasa → Glucosa 6 fosfatasa.
- Glucemia → Niveles de glucosa en sangre.
- Glucogenosis tipo I → Enfermedad de Von Gierke.
- Glucogenosis tipo II → Enfermedad de Pompe.
- Glucogenosis tipo III → Enfermedad de Cori-Forbes.
- Glucogenosis tipo IV → Enfermedad de Andersen.
- Glucogenosis tipo V → Enfermedad de McArdle.
- Glucogenosis tipo VI → Enfermedad de Hers.
- Glucogenosis tipo VII → Enfermedad de Tarui.
- Glucogenosis tipo XI → Enfermedad de Fanconi-Bickel.
- Vitamina A → Retinol
- Vitamina B_1 → Tiamina.
- Vitamina B_2 → Riboflavina.
- Vitamina B_3 → Niacina.
- Vitamina B_5 → Ácido pantoténico.
- Vitamina B_6 → Piridoxina.
- Vitamina B_8 → Conocida también como vitamina H o biotina.
- Vitamina B_9 → Ácido fólico.
- Vitamina B_{12} → Cobalamina.
- Vitamina C → Ácido ascórbico.
- Vitamina D → Calciferol.
- Vitamina E → Tocoferol.
- Vitamina K → Fitomenadiona.

AEEg
Asociación Española de Enfermos de Glucogenosis

FUNDACIÓN genzyme

La Asociación Española de Enfermos de Glucogenosis, desde su constitución en 1999, ha querido ser en la medida de sus posibilidades, un catalizador de las inquietudes e iniciativas de todos los colectivos, afectados por estas enfermedades. Igualmente, desea servir para la realización en España de actividades de información a propósito del diagnóstico, de los cuidados y de las alternativas terapéuticas, así como para la aprobación de iniciativas ya existentes en otros países de nuestro entorno.

La AEEG participa en los foros internacionales con el fin de trabajar conjuntamente con otros grupos y organizaciones que tengan relación con las Glucogenosis. La AEEG es miembro de la Internacional Pompe Association (IPA), la European Organisation for Rare Diseases (EURORDIS) y la Federación Española de Enfermedades Raras (FEDER).