Bases moleculares de las alteraciones del tejido adiposo y cambios metabólicos asociados al síndrome lipodistrófico en pacientes infectados por HIV-1

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JOSE MIGUEL GALLEGO ESCUREDO, 2012

UNIVERSIDAD DE BARCELONA

Departamento de Bioquímica i Biología Molecular

Facultad de Biología

Programa de doctorado de Biomedicina

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MEMORIA PRESENTADA POR

JOSE MIGUEL GALLEGO ESCUREDO

PARA OPTAR AL TÍTULO DE DOCTOR POR LA UNIVERSIDAD DE BARCELONA

FIRMA DE LOS DIRECTORES

Francesc Villarroya Gombau **Marta Giralt Oms**

Para mis padres y Veronique. Gracias por estar siempre ahí.

El tiempo ha pasado volando (y desgraciadamente parece que se confirma lo que dicen los que van por delante, ya que tengo la sensación de que cada vez va más rápido). Parece que fue hace unas pocas semanas o pocos meses cuando llegué a este laboratorio siendo un pardillo (por decirlo de alguna manera), sin ninguna idea muy clara y con ese tufillo que aún desprende la adolescencia que no acaba de terminar, que imaginariamente te sitúa más al centro y más alto de lo que realmente estás. Parece que no fue hace tanto cuando era el más nuevo de todos y hoy, dos rodillas menos ,unas largas obras, una boda y algún viaje después entre otras cosas, soy de los que más tiempo lleva en el laboratorio. Durante este tiempo he aprendido muchas cosas, tanto dentro como fuera, no quiero ser muy pesado con los agradecimientos individualizándolos para hacerlos más graciosos, creo que ya nos hemos reído bastante en todos estos años, y además es mejor que nos quedemos con todo eso como recuerdo, más que con una hoja escrita deprisa-y-corriendo para poder llevar la tesis a imprimir y poder depositarla a tiempo. Sería como cuando te dicen que cuentes el chiste ese de *nosequé*, y todo el mundo clava la mirada en ti y notas las expectativas tocando el límite más alto, y en ese preciso momento sabes que el chiste ya no va a hacer gracia y se va a producir un incomodísimo silencio cuando acabes, pues más o menos eso es lo que no quiero que pase con los agradecimientos. Simplemente quiero aprovechar estas breves líneas para daros las gracias a todos, los que estáis y los que habéis estado porque todos me habéis ayudado de una manera u otra, sobre todo los que más tiempo hemos pasado juntos, y espero haberos servido de ayuda o lo que sea en algún momento. Gracias a todos, de verdad.

Pero sobre todo, quiero agradeceros a Marta y Francesc, que me dierais la oportunidad de venir a este grupo, que me hayáis ayudado tanto en estos años. Muchas gracias!

CONTENIDO

Esta tesis doctoral se estructura según la normativa vigente para las tesis con formato “por artículos” establecido por la Facultad de Biología de la Universidad de Barcelona y por lo tanto consta de la siguiente estructura:

1. Introducción general
2. Objetivos
3. Resumen global y discusión general
4. Conclusiones
5. Bibliografía
6. Publicaciones

- Differential effects of Efavirenz and Lopinavir/Ritonavir on human adipocyte differentiation, gene expression and release of adipokines and pro-inflammatory cytokines

- Effects of nevirapine and efavirenz on human adipocyte differentiation, gene expression, and release of adipokines and cytokines

- Differential molecular signature of visceral adipose tissue alterations in HIV-1-associated lipodystrophy

- Differential gene expression indicates that “buffalo hump” is a distinct adipose tissue disturbance in HIV-1-associated lipodystrophy

- Hypertrophied facial fat in an HIV-1-infected patient after autologous transplantation from “buffalo hump” retains a partial brown fat-like molecular signature

- Reduced levels of serum FGF19 and impaired expression of receptors for hormonals FGFs in adipose tissue from HIV-1-infected patients.

- Serum FGF21 levels are elevated in association with lipodystrophy, insulin resistance and biomarkers of liver injury in HIV-1-infected patients

7. Informe del director de tesis sobre los artículos publicados

8. Índex

9. Apendix

- A study of fatty acid binding protein 4 in HIV-1 infection and in combination antiretroviral therapy-related metabolic disturbances and lipodystrophy

- Adipogenic/Lipid, Inflammatory, and Mitochondrial Parameters in Subcutaneous Adipose Tissue of Untreated HIV-1–Infected Long-Term Nonprogressors: Significant Alterations Despite Low Viral Burden

- Genetic and Functional Mitochondrial Assessment of HIV-Infected Patients Developing HAART-Related Hyperlactatemia

- Histological and molecular features of lipomatous and nonlipomatous adipose tissue in familial partial lipodystrophy caused by LMNA mutations

- Lipotoxicity on the Basis of Metabolic Syndrome and Lipodystrophy in HIV-1- Infected Patients Under Antiretroviral Treatment

- Nadir CD4 T Cell Count as Predictor and High CD4 T Cell Intrinsic Apoptosis as Final Mechanism of Poor CD4 T Cell Recovery in Virologically Suppressed HIV-Infected Patients: Clinical Implications

- Uridine Metabolism in HIV-1-Infected Patients: Effect of Infection, of Antiretroviral Therapy and of HIV-1/ARTAssociated Lipodystrophy Syndrome

INTRODUCCIÓN GENERAL

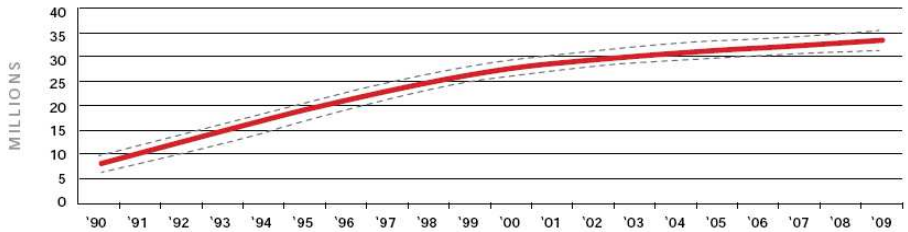
1. VIRUS DE LA INMUNODEFICIENCIA HUMANA (HIV) Y SIDA

1. A. INTRODUCCIÓN Y PANDEMIA

El virus HIV (Virus de la Inmunodeficiencia Humana) es un retrovirus que fue identificado en 1983 como el agente causal del Síndrome de la Inmunodeficiencia Adquirida (SIDA) que a su vez se había descrito dos años antes [1-3]. La infección, que principalmente se da por vía sexual o parenteral, de este virus, se ha ido propagando a nivel mundial desde su descubrimiento, hasta el día de hoy sin que los esfuerzos que se han realizado hayan servido para obtener una vacuna o una cura. A pesar de ello, dichos esfuerzos no han sido en vano, ya que parte de ellos han derivado en un tratamiento conocido como HAART (*Highly Active Antiretroviral Therapy*) gracias al cual se ha logrado convertir la infección en una enfermedad crónica en vez de una mortal [4], al menos en los países desarrollados en los que los infectados tienen acceso completo a los medicamentos que forman parte de esta terapia.

Actualmente se calcula que la población mundial de afectados por el virus, asciende aproximadamente a 35 millones de los cuales el 50% son hombres. Esta cifra parece haberse estabilizado tras dos décadas de alarmante ascenso. (Figura 1) El continente africano, lugar de origen del virus, presenta una altísima incidencia de la infección con casi 25 millones de infectados, superando el 70% del total. El 34% de los infectados totales son habitantes de países de la zona sudafricana, indicando que la zona sur del continente africano soporta gran parte de la carga de la pandemia (datos obtenidos del informe de UNAIDS en su *Global Report* 2010). Se considera la epidemia del HIV como la más mortífera con más de 25 millones de muertos hasta nuestros días [5].

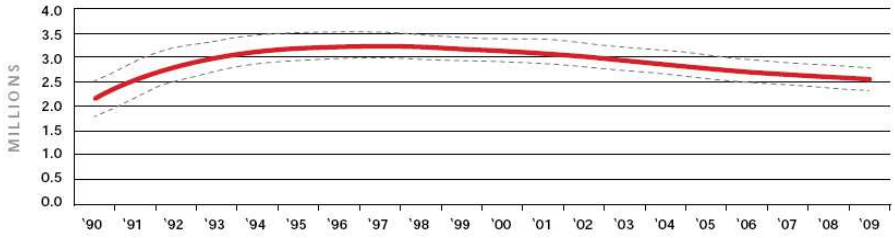
Figura 1. Numero total de personas infectadas por el virus por año. Adaptado de UNAIDS 2010 Global Report.



A pesar de esto, las mismas fuentes, recogen un esperanzador descenso de un 19% a nivel global en la incidencia de infección por HIV desde 1999 (año que marcó el pico máximo de incidencia) y 2009 (figura 2). Esta significativa reducción superó el 25% en 33 países, 22 de los cuales eran subsaharianos con altas tasas de infecciones. En contraste con estos optimistas datos aparecen los datos de algunos países de la región

de Europa del este y Asia central, que en el mismo periodo de tiempo han sufrido incrementos globales de incidencia de infección por el virus de más del 25%. En estos países el uso parenteral de drogas sin acceso a agujas estériles parece ser una de las principales razones del aumento.

Figura 2. Numero de nuevos infectados por el virus por año. Adaptado de UNAIDS 2010 Global Report.



Del mismo modo las muertes provocadas por la infección por el HIV presentan una clara tendencia al descenso en todas las regiones menos en las ya comentadas Asia central y Europa del este. (UNAIDS 2010 *Global Report*)

1. B. VIRUS DE LA INMUNODEFICIENCIA HUMANA (HIV)

1. B. 1. ORIGEN Y ESTRUCTURA DEL VIRUS

El Virus de la Inmunodeficiencia Humana (HIV) es un retrovirus del género *Lentivirus* de la familia *Retroviridae*. Se han descrito dos especies capaces de infectar humanos: HIV-1 y HIV-2, que a su vez se dividen en varias subespecies. El HIV-1 se divide en 4 subespecies: M, N, O y P. Y por su parte el HIV-2 se divide en 8 subespecies cuya nomenclatura abarca desde la A hasta la H [6]. La subespecie culpable de la pandemia global con una mayoritaria distribución mundial es la subespecie HIV-1 M [5]. El HIV-2 es menos infectivo y presenta una progresión más lenta que la especie 1, y su distribución se limita a la zona del este del continente africano [6-8]. Por esto la cepa HIV-1 M es la que se asocia comúnmente al desarrollo del SIDA.

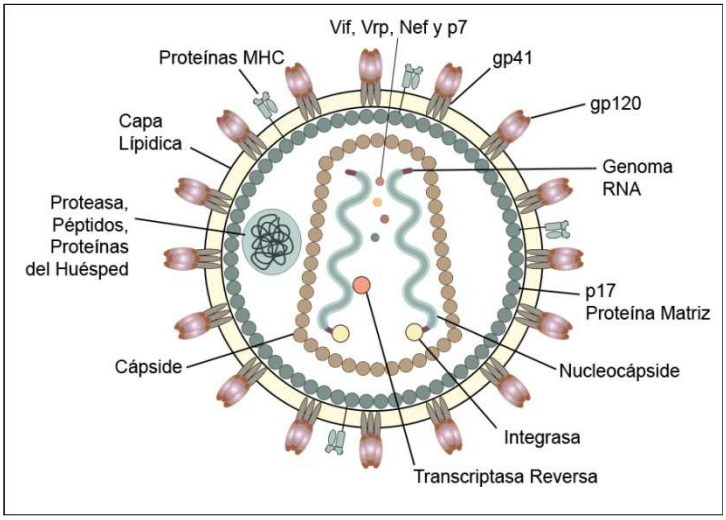
Ambas especies de virus no parecen compartir un antecesor común, ya que filogenéticamente las subespecies M,N y O del HIV-1 está altamente relacionado con el SIV (*Simian Immunodeficiency Virus*) que infecta chimpancés (SIVcpz) [9-11], y por su parte la subespecie P, descubierta en 2009 y solo presente en personas con origen camerunés [12] parece proceder del SIV que aparece en algunos gorilas (SIVgor) de la zona de Camerún [12]. Por otro lado, la especie HIV-2 está relacionada con el SIV que

infecta al simio mangabeye gris (SIVsm)[13;14]. El hecho de que el virus SIV no provoque SIDA en estos simios, hace pensar en una zoonosis como explicación en la cual estos simios sirvan de reservorio para el virus [11]. Este hecho se ve reforzado por la presencia de un SIV que infecta macacos (especie de simios asiáticos) en cautividad y que, en estos simios, sí provoca SIDA [15;16]. Se ha descrito que el SIV que infecta estos macacos procede del mangabeye gris, hecho que sugiere que la infección inter-especie si es capaz de provocar SIDA, reforzando la idea de la zoonosis [16].

No existen muchas evidencias sobre cómo y cuando el virus logró una infección inter-especie. Todo parece indicar que cada una de las subespecies proviene de transmisiones a humanos independientes [6]. La distribución original de las subespecies del HIV coincide con la distribución de sus antecesores filogenéticos que infectan los simios (SIV). Teniendo en cuenta esto y que la caza de estos simios es y ha sido una costumbre común en África, la teoría más aceptada afirma que las mordeduras y contactos entre los simios y los cazadores, han podido ser el origen de las transmisiones [17].

La estructura de los HIV ha sido bien descrita (figura 3) y es importante para comprender su patogénesis. Como todos los Lentivirus son retrovirus que se caracterizan por poseer genomas complejos y una cápside con forma cónica. Un virión maduro de HIV-1 presenta un diámetro aproximado de 100nm y su bicapa lipídica es obtenida por gemación de la célula huésped. Esta bicapa lipídica presenta las glicoproteínas víricas gp120 en su superficie (SU) que aparecen acompañadas por proteínas de membrana de la célula huésped como proteínas HLA de clase I y II o proteínas de adhesión como ICAM-1, que pueden facilitar la entrada del virión a otras células diana[18]. Las gp120 aparecen unidas a unas proteínas transmembrana (TM) conocidas como gp41. La parte interior de la bicapa lipídica aparece cubierta por la proteína p17 que forma una matriz (MA) icosaédrica [19]. Protegiendo el material genético del virus aparece la cápside (CA) cónica, compuesta por unas 2000 copias de la proteína p24. Dentro de la cápside encontramos dos copias de RNA unido a unas 2000 copias de la proteína de nucleocápside (NC) p7 [19;20]. Encapsuladas junto al material genético en la cápside, encontramos 3 enzimas esenciales codificados por el virus, como son la Proteasa (PR) p11, la transcriptasa reversa (RT) p66/p55[21;22] y la integrasa (IN) p32[23], además de algunas proteínas accesorias como Nef, Vif o Vpr. Otras proteínas accesorias como Rev, Tat o Vpv no son empaquetadas en la cápside [19;20].

Figura 3. Estructura de un virión maduro y esquema de sus principales componentes.



En cuanto al genoma del virus HIV-1, se puede encontrar de dos maneras: encapsulado en la cápside del virión de forma que, como se ha comentado anteriormente, aparecen dos copias en forma de RNA dimerizado casi idénticas [24] o integrado en el DNA de la célula huésped en forma de doble cadena de DNA en lo que se conoce como provirus.

Este DNA aparece protegido en ambos extremos 5´y 3´ por secuencias repetitivas LTR (*Long Terminal Repeats*) y codifica para 9 genes. Tres de ellos son las proteínas estructurales Gag, Pol y Env que son comunes en todos los retrovirus. Hay 2 genes que codifican para proteínas reguladoras como son Tat y Rev; y los 4 restantes codifican para proteínas accesorias Vpu, Vpr, Vif y Nef[25]. La única diferencia con el genoma del HIV-2 es que el gen Vpu es sustituido por uno equivalente denominado Vpx.

1. B. II. CICLO Y PATOGENESIS

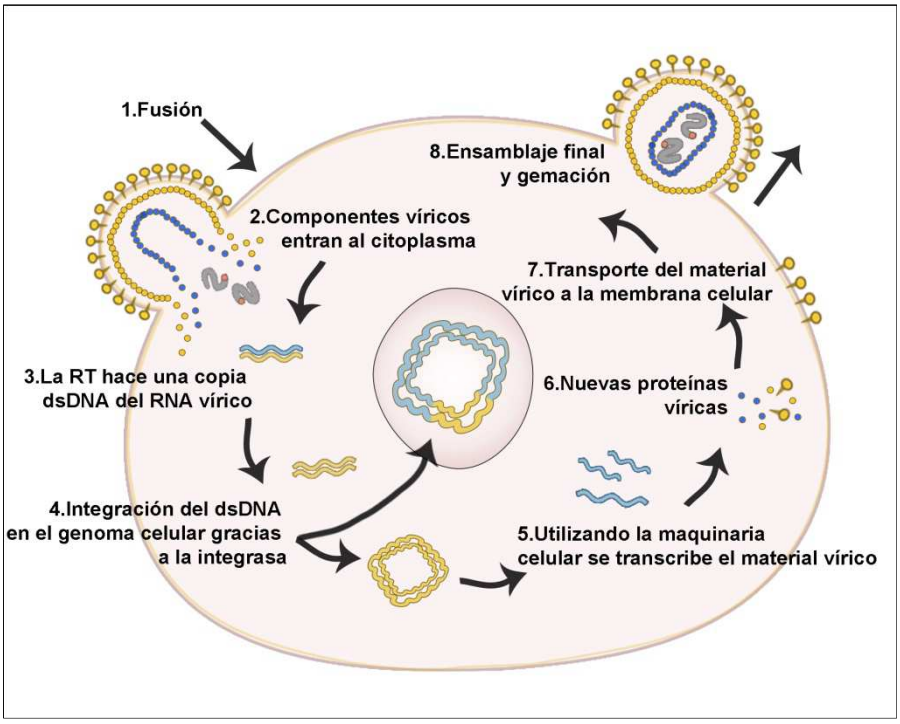
El ciclo del HIV-1 es complejo y el virus pasa por diferentes fases que conllevan diversas modificaciones estructurales. El primer paso del proceso es el reconocimiento y la unión a la célula diana, que debe ser una célula CD4+. Una vez el virus se ha unido a la futura célula huésped se da la fusión y la liberación de la cápside al citosol de la célula infectada. Una vez dentro, se dan la retrotranscripción y la integración, tras las cuales se produce la síntesis vírica, la encapsidación y la extrusión de los nuevos viriones con capacidad para infectar nuevas células diana.

Las dos moléculas principales implicadas en la **unión** del virión a la célula huésped son la glicoproteína de membrana gp120 y la transmembrana gp41[26],que

asociadas no covalentemente forman un complejo trimérico conocido como Env[27]. La gp120 es la encargada de unirse al receptor CD4 (*cluster of differentiation 4*) lo que induce cambios conformacionales que provocan la exposición de una región de gp120 que permanecía oculta, capaz de interactuar con los correceptores CCR5 o CXCR4, hecho que marca el inicio del proceso de fusión[28;29].

La **fusión** es el proceso mediante el cual la membrana plasmática de la célula huésped y el envoltorio lipídico del virus se unifican permitiendo la entrada de la cápside vírica al plasma celular. Los cambios conformacionales provocados durante la unión en gp120 y gp41, son los responsables de la fusión de las membranas[29;30]. Especialmente parece ser gp41 la encargada de funcionar a modo de ancla para permitir la aproximación de las bicapas lipídicas, permitiendo la entrada de la cápside al plasma de la célula huésped[31;32].

Figura 4. Esquema del ciclo del virus HIV en el que se detallan todas las fases desde la unión virión a la célula, hasta la formación de nuevos viriones.



Tras la fusión se da la **liberación de la cápside** al citoplasma de la célula huésped. La cápside debe ser desmantelada para que el material genético del virión pueda ser retrotranscrito. No está aún claro el mecanismo que regula el desacople de las proteínas de la cápside, pero parece que la ciclofilina A (Cyp-A) está relacionada con el desensamblaje de la capside[33].

Una vez el material genético es liberado de la cápside al citoplasma celular comienza la **retrotranscripción**. La Transcriptasa reversa (RT) asociada a otras proteínas víricas en un complejo conocido como *Reverse Transcription Complex* (RTC)[34], lleva a cabo la retrotranscripción convirtiendo las dos cadenas de RNA a una de DNA de doble cadena (dsDNA)[35]. Cabe mencionar que este punto es diana de los fármacos pertenecientes a la terapia HAART conocidos como Inhibidores de Transcriptasa Reversa (RTI), que juegan un papel muy importante en esta terapia, tal y como se expondrá más adelante.

El siguiente paso es la **integración** del dsDNA en el DNA de la célula huésped. Para ello es necesario la formación del Complejo de Preintegración (PIC), que está constituido por proteínas víricas nucleofílicas como las proteínas de nucleocápside (NC), las de la Matriz (MA)o la Vpr, la RT que también forma parte de este complejo, la integrasa vírica (IN) y enzimas de reparación del DNA del huésped que acompañan al dsDNA[36;37]. Este complejo es transportado hasta el núcleo y una vez allí la integrasa vírica cataliza cortes en ambos extremos 3´ LTR de la dsDNA vírica para preparar su integración en el DNA de la célula huesped[38]. Tras esto el dsDNA vírico es integrado en el DNA de la célula huésped por medio de la integrasa vírica. Este hecho viene facilitado por una integrasa de la célula huésped llamada LEDGF/p75 (*Lens Epithelium-Derived Growth Factor*) que uniéndose a la IN estimula la integración[39-42].

Una vez el material génico vírico se encuentra integrado en el DNA de la célula huésped, es conocido como provirus. Desde ese momento comienza la **síntesis vírica** de nuevos viriones. El DNA vírico es transcrito a RNA viral del tamaño completo y a mRNA de diferentes tamaños que se traducirán en el citoplasma celular para dar las diferentes proteínas víricas necesarias para obtener nuevos viriones. La transcripción y la traducción son llevadas a cabo por la maquinaria de la célula huésped [43;44]. Las proteínas víricas que se traducen en este proceso son: el precursor poliproteico Gag que posee 4 dominios que son MA, CA, NC y p6; el precursor poliproteico Gag-Pol del cual se obtienen 3 proteínas importantes como son PR, RT e IN; y el precursor glicoproteico Env o gp160 el cual, proteolizado por proteasas propias del huésped en el aparato de Golgi, da gp120 y gp41[34].

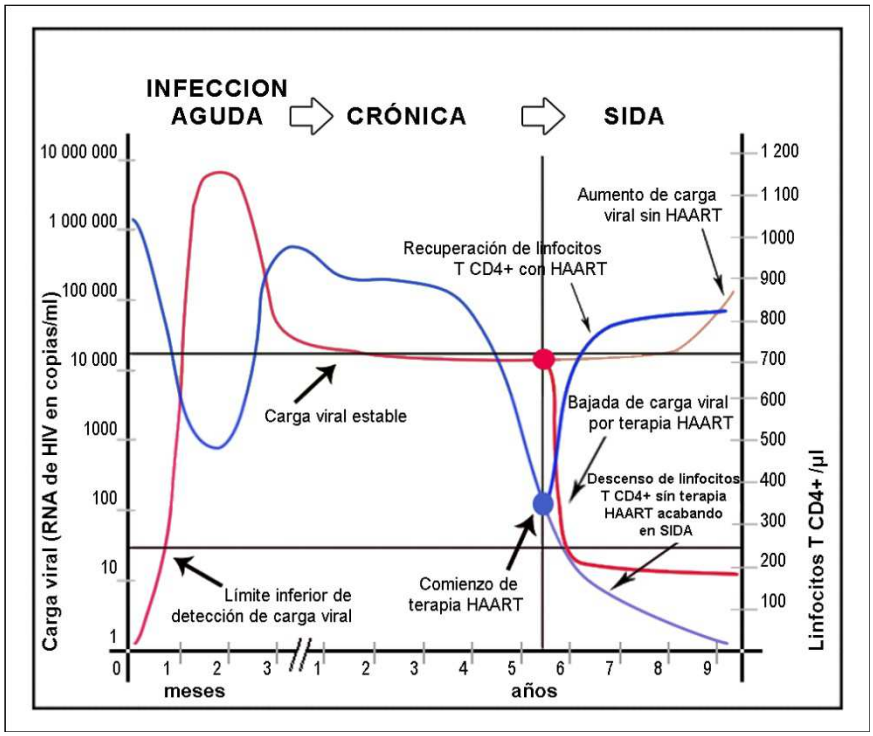
Todas estas proteínas y el RNA viral, son transportados hasta el lugar de la membrana celular donde se llevara a cabo la **encapsidación** de los nuevos viriones para su **extrusión**. El proceso de ensamblaje está dirigido por Gag, que coordina la incorporación de cada uno de los componentes virales así como de algunos factores de la célula huésped[45]. Una vez todos los componentes necesarios están en el lugar indicado, por gemación, el virión se libera de la célula huésped. Para ello, el dominio p6 de Gag secuestra componentes de la maquinaria endosomal celular[46;47]. Mientras se da la extrusión la proteasa vírica (PR) proteoliza los precursores Gag y Gag-Pol en sus respectivos dominios proteicos que permiten la maduración del virión[47].

En cuanto a la patología provocada por la infección por el HIV-1, cabe mencionar el SIDA o Síndrome de Inmunodeficiencia Adquirida, que se desarrolla en los pacientes tras un tiempo variable de haber sido infectados por el virus. El SIDA se caracteriza por un descenso notable de los Linfocitos T CD4+ lo cual provoca que el paciente no sea capaz de tener una respuesta inmune, hecho que deriva en múltiples infecciones oportunistas[48;49].

Tras la infección se da una fase en la que aumentan los niveles del virus HIV en sangre en pocas semanas, en lo que se conoce como fase aguda. En la primera parte de esta fase aguda se da la infección, en la mayoría de casos por las típicas vías sexual o parenteral. Cuando la infección se da por vía parenteral el virus entra directamente en el torrente sanguíneo, en cambio si la infección se da por vía sexual, el virus debe hacer frente a las mucosas. Las células dendríticas (DC) están presentes en las mucosas de las zonas proclives de contacto sexual como la vagina, ano o esófago y faringe[50]. Estas células serían las primeras en entrar en contacto con el HIV y de esta manera, posteriormente extenderían el virus entre los linfocitos T CD4+ y macrófagos, dándose la primera amplificación del virus a nivel linfático. El tiempo de vida medio de un virión es de 30 minutos y el número de viriones producidos en una persona, puede llegar a ser de unas 10^{10} partículas víricas por día. Las vías por las cuales el virus podría transmitirse de las DC a los linfocitos T CD4+ y macrófagos podría ser en *trans* a través de exosomas procedentes de las células dendríticas[51] o en *cis* por unión directa del virus a los receptores de manosa[52;53].

Una vez los viriones o los linfocitos T infectados se encuentran en el torrente sanguíneo se da la segunda parte de la fase aguda, la amplificación. En esta etapa se da una infección de todas las células que sean susceptibles y se observan tanto un pico en la cantidad de virus en plasma como un marcado descenso de linfocitos T CD4+. En este estado se pueden manifestar los primeros síntomas clínicos. El final de la fase aguda viene marcado por la producción de anticuerpos para el HIV-1[54].

Figura 5. Acontecimientos que ocurren a nivel de carga viral (línea roja) medido en copias de RNA de HIV /ml y linfocitos T CD4+ (línea azul) medido en linfocitos T CD4+/µl, a lo largo de las fases de infección por parte del virus HIV en presencia o ausencia de terapia HAART.



Tras estos acontecimientos se da la fase de latencia (o crónica) que se caracteriza por la ausencia de sintomatología. La viremia es más baja que durante la fase aguda a pesar de que la replicación es constante, en parte debido a la respuesta específica contra el HIV de los linfocitos-T CD8+ citotóxicos y en parte debido al descenso de células diana. La cantidad de linfocitos CD4+ continúan menguando hasta niveles muy bajos que marcarían el inicio del SIDA propiamente dicho[48]. Esta fase presenta alta variabilidad en su duración dependiendo del paciente. Puede llegar a durar de 8 a 10 años.

La resistencia a la infección por el virus es muy rara, pero existen 2 tipos de pacientes que presentan inmunidad o alta resistencia a la infección por HIV-1. Se trata de los LTNP (*Long terminal Non Progressors*) y los HEPSN (*Highly exposed persistently seronegative)* respectivamente[55]. Sus mecanismos de inmunidad innata y adquirida están siendo estudiados con especial interés de cara a lograr una vacuna contra este virus[56].

Cuando el paciente es tratado con la terapia HAART, los niveles de RNA víricos disminuyen hasta niveles indetectables, y por el contrario se recuperan los niveles de linfocitos CD4+[57].

2. TERAPIA HAART Y TOXICIDAD ASOCIADA

2. A. INTRODUCCIÓN

Tras el descubrimiento de que el virus HIV-1 era el culpable de la pandemia que estaba provocando la inmunodepresión de un alto número de pacientes ocasionando irremediablemente su muerte, se comenzó a trabajar en la búsqueda de una solución. Quedando lejos la posibilidad de encontrar una vacuna, que aún hoy en día se busca sin éxito, aparecieron los primeros fármacos de la terapia antirretroviral a finales de los 80[58]. Estos primeros fármacos, administrados en mono o bi-terapia, mostraron poca eficacia ya que eran incapaces de controlar el virus y suprimir la replicación viral. Tuvieron que pasar algunos años, hasta que a mediados de los 90 se aprobaron nuevos compuestos que pertenecían a familias farmacológicas novedosas. Este hecho marcó el comienzo de la terapia HAART (*Highly Active Antiretroviral Therapy*).

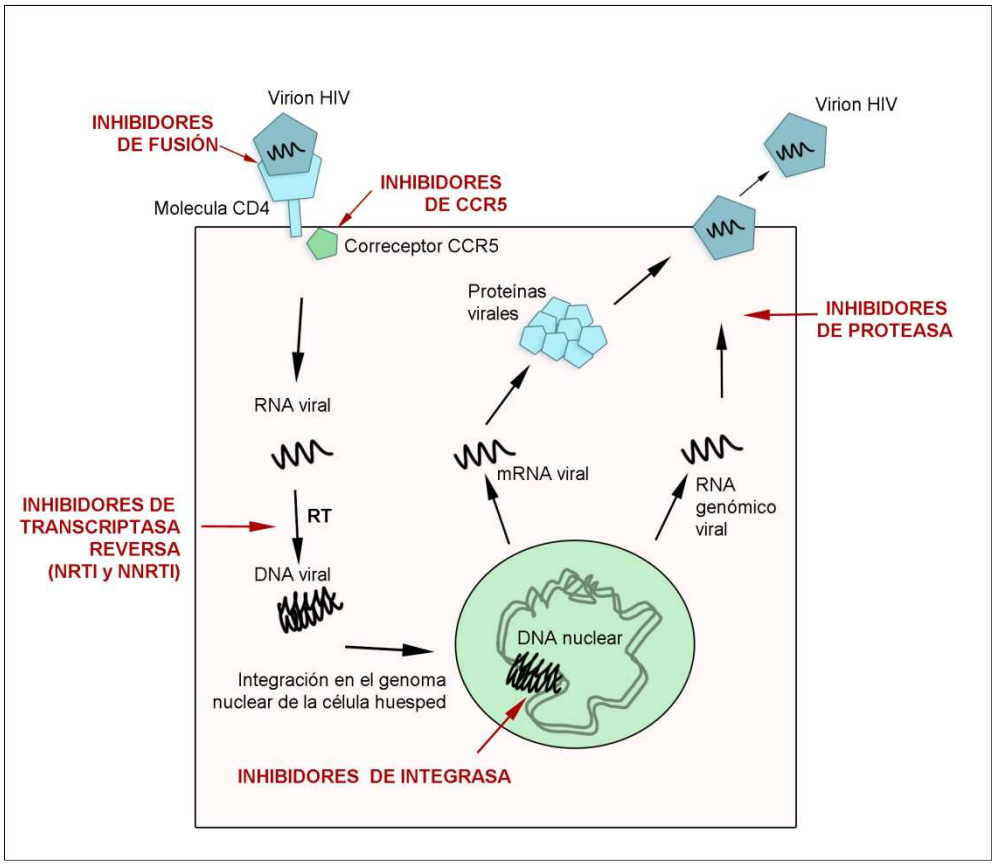
La base de la capacidad de esta terapia para suprimir casi completamente la replicación viral, radica en las combinaciones que se realizan utilizando fármacos de las diferentes familias que componen la terapia y que actúan a diferentes niveles del ciclo vírico multiplicando su capacidad de controlar del virus. De esta manera cada una de las familias que componen la terapia es capaz de inhibir enzimas esenciales en el ciclo viral por separado y actuando conjuntamente logran una eficacia mayor inhibiendo tanto la replicación como la maduración o la entrada del virus en la célula huésped. Gracias a esta estrategia la HAART es capaz de reducir los niveles de RNA viral en plasma y de recuperar los niveles de células CD4+[59], así como recuperar la capacidad de responder a antígenos de estas células y las CD8+, que consecuentemente ha derivado en la desaparición de infecciones secundarias. Todo esto ha transformado la infección en una enfermedad crónica en vez de una mortal, lo cual ya es un gran logro[60;61].

Existen 6 familias de medicamentos que componen la terapia HAART y que actúan en 5 dianas clave del ciclo de replicación viral. Existen dos tipos de inhibidores de transcriptasa reversa vírica, los análogos de nucleósidos (NRTI, *Nucleoside Reverse Transcriptase Inhibitors*) y los no análogos (NNRTI, *Non Nucleoside Reverse Transcriptase Inhibitors*), que bloquean la retrotranscripción del material genético del virus. Otro enzima diana para otra familia de fármacos es la proteasa vírica, sobre la cual actúan los inhibidores de proteasa vírica (PI, *Protease Inhibitors*), impidiendo la maduración de las proteínas víricas ya traducidas. Estas tres familias de fármacos componen los medicamentos más clásicos de la terapia HAART.

En los últimos años se han desarrollado diferentes fármacos basados en mecanismos que bloquean dianas diferentes a las clásicas. Si bien no son aún primeras elecciones en las pautas preferenciales de administración de la terapia HAART, van

ganando peso. Uno de los medicamentos con los cuales se ha logrado bloquear el funcionamiento de una nueva diana enzimática, la integrasa vírica, son los inhibidores de integrasa (II, *Integrase Inhibitors*). Estos fármacos actúan sobre la integrasa, impidiendo que lleve a cabo su función de integrar el dsDNA vírico en el genoma de la célula huésped. Por otra parte también existen los inhibidores del correceptor CCR5 (CCR5-I) que evitan de esta manera que el virus pueda penetrar en las células CD4+[62]. Del mismo modo últimamente han aparecido fármacos capaces de bloquear la fusión del virus con la célula huésped (FI, *Fussion Inhibitors*).

Figura 6. Dianas de las diferentes familias de fármacos que componen la terapia HAART a lo largo del ciclo replicativo de virus HIV en la célula T CD4+ del huesped.



A pesar del éxito de esta terapia y su capacidad para mantener las copias de RNA viral en plasma muy bajas (por debajo de les 50 copias, limite de detección) y la recuperación del sistema inmune con los linfocitos CD4+[57;63], su uso resulta obligatoriamente continuado, y con los años se han observado diferentes efectos adversos asociados a la terapia HAART. Existe controversia sobre la aportación de cada uno de los fármacos a los efectos adversos, ya que depende de diferentes factores entre los que se encuentra la variabilidad interindividual, tanto de los valores del

medicamento en plasma como de la respuesta a este. Otro hecho que dificulta aclarar el peso de cada medicamento en los efectos adversos es que los fármacos se administren de forma combinada. Hoy en día se siguen realizando estudios para determinar la aportación de cada fármaco a estos efectos secundarios no deseados.

Tabla 1. Familias de fármacos antirretrovirales utilizadas para el tratamiento de la infección por HIV (terapia HAART). En gris los fármacos de nueva generación.

FAMILIA FARMACOLOGICA	NOMBRE DEL FARMACO	MECANISMO DE ACCIÓN
NRTI	ABACAVIR (ABC)	Sustratos análogos de la transcriptasa reversa que inhiben su funcionamiento de forma competitiva interrumpiendo la síntesis del DNA viral.
	DIDANOSINA(ddI)	
	EMTRICITABINA (FTC)	
	ESTAVUDINA (d4t)	
	LAMIVUDINA (3TC)	
	TENOFOVIR DISOPROXIL FUMARATO (TDF)	
	ZALCITABINA (ddC)	
	ZIDOVUDINA (AZT)	
NNRTI	DELAVIRIDINA (DLV)	Inhibidores no competitivos de la transcriptasa reversa vírica, su mecanismo consiste en provocar cambios conformacionales en la enzima.
	EFAVIRENZ (EFV)	
	ETRAVIRINA (ETR)	
	NEVIRAPINA (NVP)	
	RILPIVIRINA (RPV)	
PI	AMPRENAVIR (APV)	Sustratos análogos de la proteasa vírica que una vez se unen a su centro activo la bloquean evitando la maduración de las proteínas víricas.
	ATAZANAVIR (ATV)	
	DARUNAVIR (DRV)	
	FOSAMPRENAVIR (fAPV)	
	INDINAVIR (IDV)	
	LOPINAVIR (LPV)	
	NELFINAVIR (NFV)	
	RITONAVIR (RTV)	
	SAQUINAVIR (SQV)	
	TRIPANAVIR (TPV)	
FI	ENFUVIRTIDA (ENF)	Evita la fusión de la cubierta viral con la de la membrana de la célula huésped.
II	RALTEGRAVIR (RGV)	Inhibidor de la integrasa vírica, evitando así la integración del DNA vírico en el genoma de la célula huésped
CCR5-I	MARAVIROC (MRC)	Bloquea la interacción virus-correceptor CCR5, evitando la fusión del virus con la célula huésped.

2. B. FAMILIAS DE FARMACOS DE LA HAART

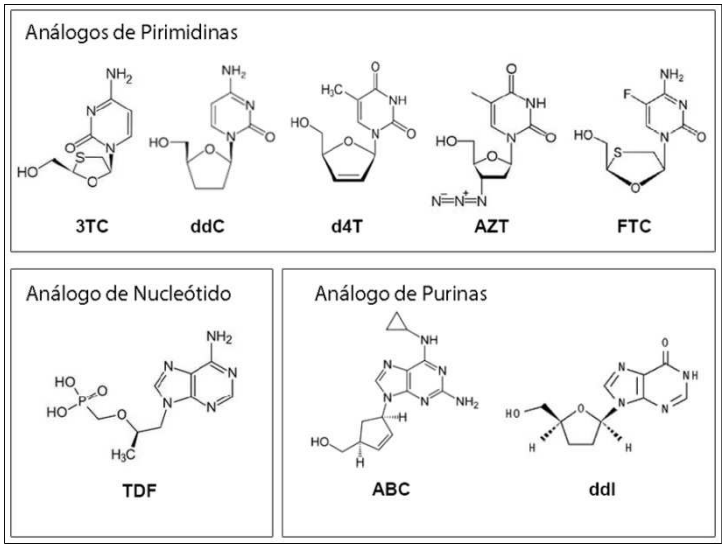
2. B. I. INHIBIDORES DE TRANSCRIPTASA REVERSA ANALOGOS DE NUCLEOSIDOS (NRTI)

Fueron los primeros fármacos aceptados en la lucha contra el HIV-1, siendo el primero el AZT (Zidovudina)[58]. Se trata de derivados de Adenosina, Guanosina, Citosina y Timina, que son considerados pro-fármacos ya que deben ser fosforilados una vez penetran en las células, generándose la forma 5`-trifosfato para ser activos. De esta

manera, son capaces de inhibir competitivamente el proceso de polimerización llevado a cabo por la transcriptasa reversa, ya que son un sustrato alternativo[64]. Su mecanismo de bloqueo del proceso de retrotranscripción, se basa en que carecen de extremo 3´OH o lo tienen modificado, de manera que impiden la formación del enlace fosfodiester 3´-5´ evitando la elongación de la cadena de DNA.

Hoy en día hay 8 fármacos aprobados de esta familia y existe variabilidad en la tolerancia que presentan los pacientes así como en su eficacia.

Figura 7. Estructuras químicas de los NRTI aprobados para terapia HAART.



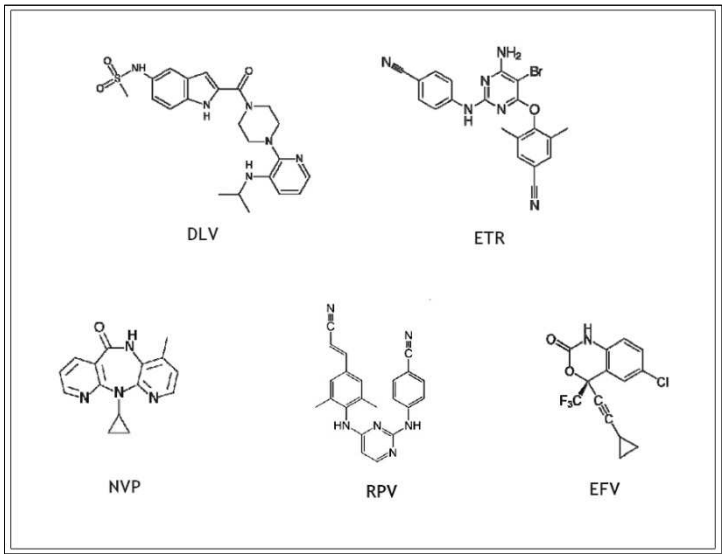
2. B. II. INHIBIDORES DE TRANSCRIPTASA REVERSA NO ANALOGOS DE NUCLEOSIDOS (NNRTI)

A pesar de actuar al mismo nivel que los anteriores, es decir bloqueando la transcriptasa reversa, su mecanismo es diferente. En este caso la inhibición del enzima es alostérica no competitiva ya que no se unen al dominio activo sino a una cavidad adyacente hidrofóbica cercana al sitio catalítico del enzima, provocando un cambio conformacional que evita el correcto funcionamiento del enzima[65]. A diferencia de los NRTI no necesitan ser modificados para ser activos.

Se caracterizan por tener una larga vida media en plasma, lo que contribuye a facilitar la dosificación. Se metabolizan vía citocromo p450 y para cada compuesto hay diferentes isoenzimas implicados en esta metabolización, lo que provoca que sus niveles plasmáticos puedan variar o que existan interacciones con otros fármacos que comparten la misma vía. Se considera que el virus es capaz de generar resistencia con

cierta facilidad a estos compuestos[66]. Hoy en día hay 5 medicamentos aceptados, pero hay varios en distintas fases de desarrollo clínico.

Figura 8. Estructuras químicas de los NNRTI aprobados para terapia HAART.

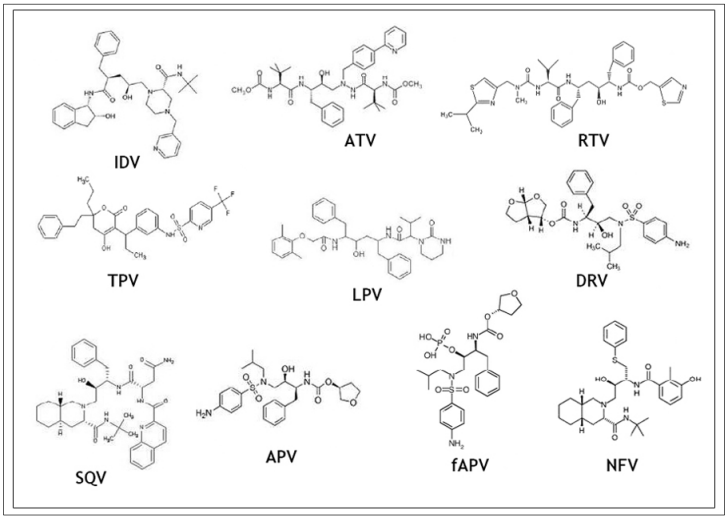


2. B. III. INHIBIDORES DE PROTEASA

La proteasa vírica se encarga de la maduración de las proteínas del virus para que sean funcionales, hecho que la convierte en una diana interesante para una terapia antirretroviral. Los PI se asemejan a las poliproteínas sustrato de esta enzima y por lo tanto actúan como sustratos análogos bloqueando el centro activo de la proteasa. De este modo se impide uno de los pasos importantes en el ciclo de replicación vírico y se bloquea la generación de nuevas partículas víricas[67].

En muchos casos se administran acompañados con una baja dosis de otro PI, el Ritonavir (RTV) debido a que este hace de potenciador de estos fármacos ya que inhibe el citocromo P450 en hígado e intestino aumentando la vida media de los PI y otros fármacos.

Figura 9. Estructuras químicas de los PI aprobados para terapia HAART.



2. B. IV. INHIBIDORES DE LA FUSIÓN

El único fármaco de esta familia aceptado hoy en día es la enfuvirtida. Su mecanismo implica la unión a la proteína gp41 de la cubierta vírica. Esta unión impide que el virus, una vez unido a la célula huésped, provoque los cambios conformacionales desencadenados por esta proteína (gp41). De esta manera se impide que las dos bicapas lipidias se fusionen y que el virión pueda penetrar en el citoplasma celular[68].

2. B. V. INHIBIDORES DEL CORRECEPTOR CCR5

Del mismo modo que en los inhibidores de fusión, a día de hoy existe solo un fármaco aceptado representando a esta nueva familia. Se trata del Maraviroc. Su diana es el correceptor CCR5. De esta manera se busca bloquear la unión y la fusión del virión con la célula huésped ya que este correceptor de la célula huésped está implicado en el cambio conformacional que sufre la célula huésped para facilitar la unión y fusión del virión y la célula CD4+[69].

2. B. VI. INHIBIDORES DE LA INTEGRASA

En este caso, la diana de esta familia de fármacos es la integración del DNA viral en el genoma de la célula huésped. Esta paso es crucial para que, utilizando la maquinaria del huésped, el virus se replique y realice numerosas copias. Estos fármacos bloquean específicamente la transferencia de la cadena genética viral[70]. Como en los dos anteriores casos, se trata de nuevos fármacos y por ahora solo hay un medicamento aceptado, es el raltegravir.

2. C. MODOS DE ADMINISTRACIÓN PREFERENTES PARA HAART

La base de la terapia HAART es la combinación de diferentes fármacos para optimizar el bloqueo de la replicación vírica. En general, algunos fármacos son menos utilizados que otros debido en parte a su limitada eficacia o por los efectos adversos que provocan. Aún así existen numerosas opciones de combinaciones de entre las cuales se intenta seleccionar la más adecuada en función de los requerimientos de cada individuo. Principalmente se parte de la terapia base, que incluye un NRTI y un PI como mínimo, ya que entre ambos optimizan el efecto antirretroviral evitando aparición de resistencias al atacar dos dianas diferentes. Pero esta terapia puede variar dependiendo del estado de la infección y, por supuesto, de las características de los pacientes. La pautas más aceptadas actualmente, al iniciar la terapia aconsejan la administración de 2 NRTI más un NNRTI o un PI[71;72].

Estas pautas preferentes recomiendan seleccionar entre el Tenofovir y el Abacavir como primer NRTIs. Entre la Emtricitabina o Lamivudina como segundo NRTI, y por último seleccionar un fármaco de una lista de NNRTIs y PIs que incluye: Efavirenz, Nevirapina, Atazanavir, Darunavir, Fosamprenavir, Lopinavir y Saquinavir, incluyéndose últimamente en esta lista el raltegravir como inhibidor de integrasa (Combinaciones de fármacos recomendadas para triple terapia por GESIDA, 2010). De esta manera el paciente debe tomar tres fármacos que posibilitan muchas combinaciones para hacer frente a la infección.

Desgraciadamente, las características del HIV, obligan a que la terapia HAART deba realizarse durante toda la vida. La exposición crónica a estos fármacos ha generado la aparición a lo largo de los años de diferentes efectos adversos. Hoy en día todos los fármacos antirretrovirales que forman parte de la terapia HAART presentan cierta toxicidad, cuyo nivel varia. Estos efectos adversos han hecho que se centren esfuerzos en elucidar los mecanismos de toxicidad implicados para controlarlos y diseñar nuevos compuestos.

2. D. EFECTOS ADVERSOS DE LA TERAPIA HAART

Existen diversos factores que juegan un papel importante influenciando la aparición de complicaciones en los pacientes bajo terapia HAART. Para una dosis igual, hay pacientes que no responden al fármaco mientras otros presentan toxicidad. Se ha sugerido que esta variabilidad interindividual deriva entre otras cosas de determinados polimorfismos genéticos, si bien también puede ser debida a la interacción con otros fármacos y la dieta[73].

Es importante tener en cuenta que los fármacos se distribuyen de manera diferente por los distintos tejidos, hecho que puede provocar que los tejidos expuestos

a mayores concentraciones de ciertos fármacos sean más propensos a mostrar toxicidad[74]. Sumado a esto, cabe señalar que los fármacos se administran de forma combinada tal y como se ha comentado anteriormente. De esta manera resulta complicado determinar el perfil toxicológico específico asociado a cada uno de los fármacos. De todas formas la toxicidad ha sido estudiada para las familias clásicas de la terapia HAART, ya que las nuevas no se asocian aún con toxicidad, y se han señalado diferentes patrones de toxicidad asociados a la familia en sí o a los fármacos específicamente.

Los **NRTI** presentan gran variedad de efectos adversos que son atribuidos normalmente a efectos tóxicos sobre la mitocondria y que varían dependiendo del tejido o el tipo celular sobre el que se observen[75]. Entre ellos cabe destacar la neuropatía periférica, la miopatía de musculo esquelético y cardiaco, pancreatitis, esteatosis hepática, ácidosis láctica, alteraciones metabólicas y lipodistrofias. Estos efectos tóxicos se asocian más a los fármacos más antiguos de la familia, exceptuando el 3TC, es decir, sobre todo al AZT, el ddI y el d4T, los cuales han sido asociados a mayor toxicidad mitocondrial[76;77].

Los **NNRTI**, por su parte se asocian a una alta incidencia en reacciones cutáneas, así como hepatotoxicidad y alteraciones metabolicas[78-81]. El EFV y la NVP, son los fármacos más ampliamente utilizados de esta familia ya que se consideran seguros y bien tolerados. Pero la administración a largo plazo presenta perfiles toxicológicos que resultan diferentes para cada uno de ellos[81]. El EFV además de las comentadas anteriormente, se asocia también a una importante toxicidad sobre el sistema nervioso[82] y una recientemente descubierta implicación en la lipoatrofia periférica[83].

Los **PI** son asociados principalmente con el aumento de riesgo de sufrir enfermedades cardiovasculares[84] y alteraciones a nivel del metabolismo de lípidos y glúcidos, como pueden ser resistencia a insulina, lipohipertrofia, lipoatrofia o dislipidemia[85-87]. A este nivel se asocian sobre todo a una acumulación de grasa a nivel visceral[86]. Los nuevos fármacos de esta familia, que se potencian con el uso de RTV, se consideran más seguros que los antiguos, pero aún así siguen asociados a un aumento de riesgo de enfermedades cardiovasculares[88].

3. TEJIDO ADIPOSO

3. A. INTRODUCCIÓN

Una de las actividades más importantes de los seres vivos, es sin lugar a dudas asegurarse una fuente de energía, que es la base para poder mantener el resto de actividades necesarias para mantenerse con vida. A lo largo de la evolución, los animales han encontrado una solución a este problema. Se trata de almacenar el excedente de energía, que se obtiene por los nutrientes que se absorben durante la alimentación, en forma de lípidos en un tejido del mesodermo, el tejido adiposo. Hasta hace pocos años se ha entendido este tejido como un simple almacén de energía, pero hoy en día se está destacando como un órgano que juega un papel crucial en la regulación y disfunción de homeostasis energética[89]. Esta homeostasis, implica un compromiso entre el aporte y el gasto energéticos que se logra gracias a una respuesta coordinada entre el sistema nervioso y el tejido adiposo. Situaciones como la ingesta, la necesidad de producir calor, el ejercicio físico o la gestación, son procesos que determinarán este balance energético, que en última instancia afectaran al peso corporal.

Un balance positivo de este equilibrio, bien sea por una ingesta excesiva o un bajo gasto energético, provoca una hipertrofia del tejido adiposo, provocando un aumento del peso del individuo que puede conducir a la obesidad. Cada día se conocen más alteraciones metabólicas asociadas a la obesidad que implican un alto riesgo para la salud como la resistencia a insulina, dislipidemias o enfermedades cardiovasculares. Estas alteraciones se conocen en conjunto como síndrome metabólico. Hoy en día en los países desarrollados debido al acceso a alimentos altamente calóricos y al sedentarismo de la sociedad en general, las tasas de obesos con respecto a los individuos delgados o de pesos normales, está llegando a números preocupantes.

Han sido identificados tres tejidos adiposos diferentes en mamíferos, el tejido adiposo blanco (*White Adipose Tissue*, WAT), tejido adiposo marrón (*Brown Adipose Tissue*, BAT) y el tejido adiposo de la medula ósea[90-92]. Los más estudiados han sido el WAT y el BAT, ambos presentan capacidad para metabolizar y almacenar lípidos, pero funcionalmente resultan muy diferentes.

En ambos casos, se almacenan triglicéridos, pero en el caso del tejido adiposo blanco, esto sirve como almacén del exceso de energía y en cambio en el tejido adiposo marrón se usa como combustible para la generación de calor mediante la disipación de energía. De esta manera el WAT es capaz de hidrolizar estos triglicéridos para liberar ácidos grasos y glicerol en situaciones de falta de sustratos energéticos como el ayuno. Además el tejido adiposo blanco es considerado un órgano endocrino. Ambos tejidos se desarrollan en lugares concretos y diferentes.

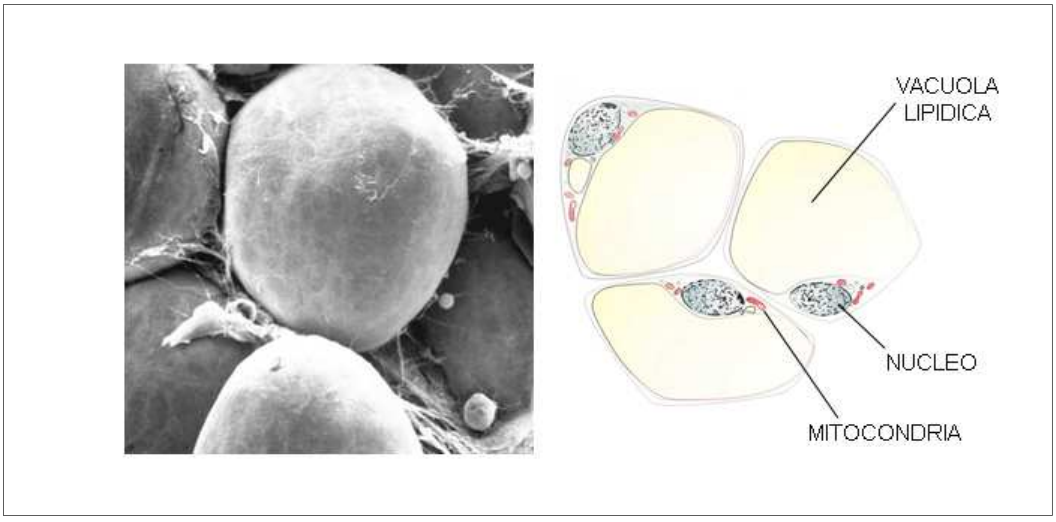
En cuanto a estas localizaciones diferenciales, cabe destacar que incluso dentro del WAT, se reconocen diferentes localizaciones (como la subcutánea y la visceral) que presentan un conjunto de características metabólicas y moleculares diferentes[93].

En cambio el papel de los adipocitos medulares ha sido poco estudiado y no se conoce en profundidad[92]. Su número y medida parece inversamente proporcional a la actividad hematopoyética de la médula ósea. El patrón de expresión de estos adipocitos se parece al de los adipocitos subcutáneos de humanos, hecho que hace pensar que sus funciones podrían ser similares[94].

3. B. TEJIDO ADIPOSO BLANCO

El tejido adiposo blanco es el tejido adiposo mayoritario y aparece distribuido en gran cantidad y en diferentes depósitos concretos (como el subcutáneo y el visceral o alrededor de algunos órganos) que presentan diferentes patrones metabólicos y de expresión génica[95;96]. Además de los adipocitos diferenciados típicos de este tejido adiposo blanco, podemos encontrar otros tipos celulares, que pueden llegar a constituir hasta el 50% del número total de células del tejido, como preadipocitos no diferenciados, fibroblastos, células endoteliales de los vasos, células del sistema inmune o células nerviosas. Las células adiposas maduras de este tejido se caracterizan por tener una gran vacuola lipídica que ocupa la mayoría del citoplasma (desplazando el núcleo a una posición lateral) y unas pocas mitocondrias con crestas poco desarrolladas.

Figura 10. Morfología típica de los adipocitos blancos.



Como se ha comentado, su función asociada a la homeostasis energética implica almacenar la energía sobrante obtenida mediante la dieta en forma de triglicéridos y liberar esta energía en forma de ácidos grasos libres y glicerol cuando es necesario. Del mismo modo cada día se conocen más factores endocrinos y paracrinos que son liberados por el tejido adiposo blanco, que juegan un importantísimo papel en la regulación metabólica de la homeostasis corporal.

Además de actuar como órgano endocrino y reservorio de energía, cumple otras funciones siendo un excelente aislante térmico así como ofreciendo una protección mecánica al cuerpo[97].

3. B. I. DIFERENCIACIÓN ADIPOCITARIA DEL TEJIDO ADIPOSO BLANCO

En humanos el tejido adiposo blanco aparece a mitad de la gestación aproximadamente, más tarde que el marrón. Su origen son las células madre mesenquimales multipotentes que tiene origen mesodérmico. Cabe destacar que los preadipocitos aislados de diferentes depósitos, como el visceral y el subcutáneo, poseen diferentes potenciales adipogénicos cuyas bases son desconocidas[98;99]. Este hecho hace que una vez maduras, las células adipocitarias tengan diferentes comportamientos metabólicos[100].

Existen dos fases en el proceso de diferenciación del tejido adiposo. La primera es la **determinación**[101]. Este proceso abarca los mecanismos por los cuales la célula madre pluripotente adquiere las características de la línea adipocitaria e implica la transformación de la célula madre a preadipocito, que morfológicamente aún será igual a su precursora. Este hecho conlleva la pérdida por parte de la célula de la capacidad para convertirse en otro linaje mesenquimal como los miocitos, condriocitos o osteocitos[102].

Tras este paso se da la **diferenciación terminal,** que se caracteriza por la adquisición por parte del preadipocito de la maquinaria proteica necesaria para el transporte y síntesis de lípidos, la sensibilidad a la insulina y la capacidad de secretar adipoquinas, convirtiéndose ya en un adipocito maduro[101;102].

Los acontecimientos que promueven la determinación permanecen aún poco claros, en cambio las vías transcripcionales de la diferenciación terminal han sido bien descritas. Para realizar estos estudios se han utilizado estudios *in vitro* con las limitaciones que esto conlleva, ya que el tejido adiposo es un sistema complicado.

Para estos cultivos celulares se han utilizado líneas celulares preadipocitarias murinas como lo son las 3T3-L1 o las 3T3-F442A[103;104] y también cultivos primarios de preadipocitos aislados de la fracción del estroma vascular del tejido adiposo disociado[102]. Aún así, hasta hace poco no existía ninguna línea celular de adipocitos humanos con capacidad de diferenciarse después de diversos ciclos de división. Pero

en 2001 aparecieron unos adipocitos aislados del tejido adiposo de unos niños que sufrían el Síndrome de Simpson-Golabi-Behnel (SGBS)[105]. Este síndrome provoca un fenotipo de hipercrecimiento generalizado. Las células obtenidas se pueden diferenciar tras más de 50 rondas de división manteniendo una morfología, funcionalidad y bioquímica idéntica a adipocitos de individuos sanos[106]. Por eso han sido y serán una herramienta muy importante en el estudio del desarrollo y el metabolismo del tejido adiposo blanco humano.

3. B. II. CASCADA DE ACTIVACION DE LA DIFERENCIACION Y FACTORES IMPLICADOS

Como se ha comentado, los pasos que siguen los preadipocitos para convertirse en adipocitos han sido ampliamente estudiados *in vitro*. Los pasos que se llevan a cabo han resultado altamente ordenados. El primer acontecimiento es la parada de la proliferación por parte de los preadipocitos proliferantes que salen de esta manera, del ciclo celular. Este hecho se debe generalmente a procesos de inhibición por contacto, a pesar de que el contacto célula-célula no parece ser indispensable[102;107]. En cultivos celulares este paso se induce con la adición de factores adipogénicos tras lo cual se dan aún una o dos rondas de división celular (expansión clonal) y se inicia la activación transcripcional de genes marcadores del adipocito, lo que conlleva a una paulatina adquisición del fenotipo adiposo.

Los acontecimientos a nivel transcripcional que tiene lugar en este punto se dan en dos fases. Durante la primera ocurre una inducción de dos factores de transcripción de la familia C/EBP (*CCAAT/Enhancer Binding Protein*) como son: **C/EBPβ y C/EBPδ**. Estos factores de transcripción son responsables del inicio de la segunda fase de diferenciación ya que activan la expresión de **PPARγ** (*Peroxisome Proliferator Activated Receptor γ*) y **C/EBPα**[108;109]. Estos dos son genes *master* del resto del proceso de diferenciación, se trata de dos factores de transcripción capaces de activar la gran mayoría de genes que caracterizan el fenotipo adipocitario y que se expresan durante esta segunda fase como son la FAS (*Fatty Acid Synthase*), la glicerofosfato deshidrogenasa, la acetil-CoA carboxilasa, el transportador de glucosa GLUT4, el receptor de insulina y la proteína aP2/FABP (*Fatty Acid Binding Protein)* específica de adipocitos) entre otros[110].

Durante la fase primera, la célula adopta una morfología esférica, más acorde con el fenotipo adipocitario y comienza a aumentar la expresión de marcadores adipogénicos como LPL (*Lipoprotein Lipase*)[108;111]. A lo largo del proceso de diferenciación van apareciendo en el citoplasma de la célula gotas lipídicas que van aumentando de volumen para después fusionarse formando una o dos grandes vacuolas lipídicas que ocupan una gran parte del citoplasma celular.

El factor de transcripción PPARγ resulta crucial para este proceso de diferenciación pero también es necesario para mantener el estado diferenciado del adipocito[112]. Por

si solo PPARγ es capaz de que se expresen la mayoría de genes adipocitarios pero C/EBPα es indispensable para que el adipocito adquiera sensibilidad a insulina[113]. Ambos factores de transcripción son capaces de autoinducirse y también se inducen el uno al otro[114].

Otros factores de transcripción que están implicados en el proceso de adipogénesis son el LXR (*Liver X Receptor*), SREBP1c (*Sterol Regulatory Element Binding Protein 1*) y CREB (*cAMP Response Element Binding Protein*).

Las C/EBPs son proteínas de unión a CCAAT/enhancer que pertenecen a la familia de los factores de transcripción bZIP (*Basic leucine Zipper*) con un dominio básico de unión a DNA y una cremallera de leucinas. Actúan como homo y como heterodimeros y su distribución tisular no se limita al tejido adiposo[115].

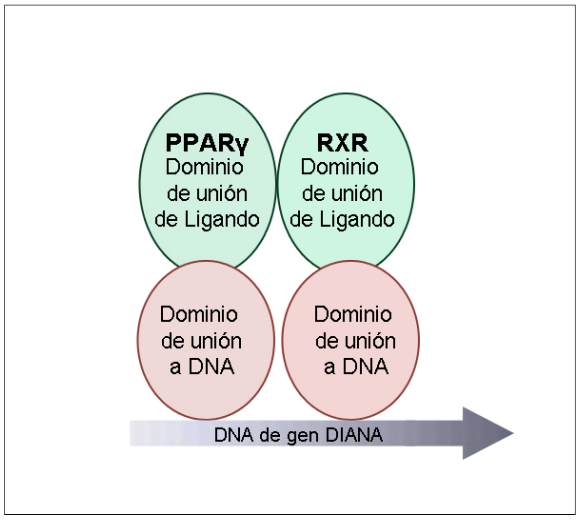
La expresión ectópica de uno de los miembros de la familia **C/EBP**, el C/EBPβ es suficiente para inducir la diferenciación de las células 3T3-L1 murinas sin ningún inductor hormonal adicional y su activación parece estar inducida vía cAMP[116]. En el caso de C/EBPδ su presencia es clave para acelerar el proceso de diferenciación[116]. De este modo la expresión ectópica de C/EBPβ y no de C/EBPδ, es capaz de determinar el linaje de adipocito las células fibroblásticas NIH 3T3 y promueve su diferenciación en presencia de inductores hormonales[117]. De esta manera los fibroblastos a los que falta una de estas dos proteínas presentan una cierta pérdida de capacidad de diferenciación adipocitaria, en cambio si les faltan ambas pierden esta capacidad de manera muy severa[118]. En ratones sin alguno de estos factores se observa un tejido adiposo blanco normal mientras en el marrón se aprecia una pérdida de acumulación lipídica, así como un descenso en la expresión de UCP1 (*Uncoupling Protein 1* específica de tejido adiposo marrón). Si no se expresa ninguno de estos factores el fenotipo es mucho más severo muriendo un 85% de los ratones en el periodo perinatal y el resto que sobrevive presenta un WAT y un BAT reducidos[118]. En este caso la reducción del BAT es debido a una disminución de la acumulación lipídica, mientras la del WAT se debe a una disminución del número de células que son normales en todos los aspectos.

La sobreexpresión de otro miembro de esta familia C/EBPα en células 3T3-L1 da lugar a su diferenciación y en cambio su bloqueo con RNA antisentido evita esta diferenciación[119;120]. Se conoce que C/EBPα se une y transactiva a muchos genes relacionados con el fenotipo adipogénico como: aP2/FABP, SCD1 (*Stearoyl-CoA Desaturase 1*), GLUT4, PEPCK, leptina y el receptor de insulina entre otros. También se le atribuye una función antimitótica[121;122]. Su deleción en homocigosis en ratones provoca una importante reducción de la acumulación de grasa en ambos tejidos adiposos[123]. Estos ratones mueren de hipoglucemia ya que no pueden realizar la gluconeogénesis en el hígado, pero si se introduce un transgen en el hígado para que

puedan realizarla, la supervivencia aumenta pero el tejido adiposo blanco no se recupera, mientras el marrón si[124;125].

El receptor activado por proliferadores peroxisomales gamma, **PPARγ** es el más adipogénico de los PPAR. Pertenece a la superfamilia de los receptores nucleares de hormonas y es un factor de transcripción que necesita heterodimerizarse con otro receptor nuclear de hormona, en este caso RXR (*Retinoid X Receptor*) para poder unirse al DNA y llevar a cabo su función transcripcional[126]. El gen PPARγ presenta dos isoformas derivadas de un splicing alternativo, la isoforma 1 y la 2[127]. La primera se expresa poco y en un amplio espectro de tejidos mientras la segunda es específica del tejido adiposo[128], estando relacionada con el metabolismo lipídico.

Figura 11. Heterodímero de PPARγ y RXR con sus característicos dominios de unión a ligando y unión a DNA.



Se ha observado que fibroblastos en cultivo infectados con retrovirus que dirigen la expresión de PPARγ son capaces de diferenciarse a adipocitos, dando una idea de la capacidad adipocitaria de este factor de transcripción[129]. *In vivo* se ha observado que PPARγ es necesario para el desarrollo y la diferenciación adipocitaria[130]. PPARγ juega un papel crucial en la expresión de genes como PEPCK o aP2/FABP[131].

Estudiando PPARγ se observó que los TZD (Tiazolidinedionas) como la troglitazona, pioglitazona y rosiglitazona, agentes antidiabéticos sintéticos, son sus agonistas[132;133]. Los TZD son fármacos con potencial para tratar la resistencia a insulina. En tejido adiposo los TZD son capaces de aumentar la sensibilidad a insulina supuestamente aumentando la diferenciación adipocitaria vía PPARγ logrando adipocitos más pequeños y más sensibles a la hormona, sobre todo en el tejido adiposo blanco[132]. Se ha observado que el tratamiento con TZDs aumenta la adipogénesis tanto In vivo como in vitro[134-136]. Además de estos compuestos están los

ligandos naturales de PPARγ, entre otros la 15 deoxiΔ^{12,14}prostaglandina J2 o 15dPGJ2, capaz de activar PPARγ provocando adipogénesis en fibroblastos en cultivo[137] u otros como ácidos grasos como el oleico o el linoleico[131;138].

En cuanto al **RXR** cabe decir que es un miembro de los receptores nucleares de hormonas que tiene 3 isoformas α, β y γ, siendo la isoforma RXRγ la preferente en tejido adiposo marrón y la RXRα en tejido adiposo blanco[139]. Estos receptores pueden homodimerizar o heterodimerizar. La heterodimerización se puede dar con diferentes receptores actuando en diversas vías. En cuanto a la acción adipogénica que nos ocupa, la heterodimerización que sucede, se da con PPARγ, como se ha comentado[127] (figura 11). El ligando de RXR es el ácido *9-cis* retinoico[140]. Se ha observado que los ligandos sintéticos de RXR son capaces de provocar efectos similares a los de los TZD sobre alteraciones metabólicas como la diabetes mellitus tipo II, del mismo modo que el tratamiento con estos agonistas potencia la diferenciación de los adipocitos blancos y marrones[141].

Otro de los factores implicados en la diferenciación adipocitaria es el **ADD1/SREBP1** (*Adipocyte Differentiation and Determination Factor 1/ Sterol Regulatory Element Binding Protein 1*). Estos factores de transcripción actúan uniéndose a los elementos de respuesta a esterol. Se induce durante la adipogénesis y se regula por procesos de ayuno y realimentación[142;143]. Parece que la insulina juega un papel importante en su activación durante la realimentación[142]. La molécula completa de este factor de transcripción es una molécula inactiva anclada a la membrana del retículo endoplasmático que tras una proteólisis se trasloca al núcleo donde lleva a cabo su cometido como factor de transcripción para diferentes genes relacionados con metabolismo de ácidos grasos y triacilgliceroles como FAS, ACO o la glicerofosfato aciltransferasa 1 y 2. Este factor también parece involucrado en la adipogénesis pero no de forma tan importante como PPARγ o C/EBPα. Su expresión ectópica induce la diferenciación de la célula adiposa probablemente induciendo la síntesis de ligandos endógenos de PPARγ[144].

3. B. III. SEÑALIZACIÓN EXTRACELULAR DE LA ADIPOGENESIS

Existen diversos factores extracelulares capaces de activar vías de señalización que en última instancia regulan los factores de transcripción que se han comentado anteriormente y que controlan la adipogénesis. Estos factores, entre los que encontramos activadores e inhibidores del proceso de adipogénesis, determinaran que los preadipocitos inicien el proceso de diferenciación o permanezcan quiescentes.

Entre los factores activadores se encuentra la **Insulina**, que promueve el almacenaje de lípidos en el tejido adiposo y que promueve la diferenciación

adipocitaria. También inhibe la lipólisis inducida por catecolaminas. En el tejido adiposo la Insulina actúa vía el receptor de IGF-1 (*Insulin-like Growth Factor 1*), IGF-1R.

Otros inductores de la adipogénesis in vitro son los **glucocorticoides** (GC). Su acción probablemente regulada a través de los receptores de glucocorticoides (GR)[145], es capaz de regular la actividad transcripcional de C/EBPβ dirigiendo la acetilación de este factor de transcripción[146]. También son capaces de inducir rápidamente C/EBPδ[116].

Otros moduladores positivos de la adipogénesis son los **ácidos grasos** mono-saturados o poli-insaturados, que parecen actuar como ligandos o precursores de ligandos de PPARγ. El **cAMP** también promueve la adipogénesis como se ha comentado anteriormente, induciendo la expresión de C/EBPβ[133]. Las **prostaglandinas** también provocan la diferenciación adipogénica de los preadipocitos. La prostaciclina (PGI_2) es un metabolito del ácido araquidónico que es capaz de estimular la adipogénesis uniéndose al receptor IP de membrana de los preadipocitos. Esta unión activa la adenilato ciclasa y el consecuente aumento de cAMP que promueve la inducción de C/EBPδ y C/EBPβ[147]. Del mismo modo la PGI_2 también parece ser un ligando PPARγ[148].

En cuanto a los inhibidores de la adipogénesis cabe destacar algunos de los más conocidos. El **ácido retinoico** es un derivado de la vitamina A obtenida en la dieta. A altas concentraciones es capaz de inhibir la diferenciación adipocitaria en las primeras fases del proceso[149] ya que en los pasos finales del proceso ya no resulta efectivo. Parece que su mecanismo de inhibición se basa en impedir la inducción de los C/EBPs[150]. La **Pref-1** (*Preadipocyte factor 1*) también es inhibidora de este proceso por mecanismos aún poco claros[151]. Las glicoproteínas de la familia **Wnt** son importantes en la inhibición de la adipogénesis. Estas glicoproteínas son capaces de unirse a receptores *frizzled* inician cascadas de señalización que inhiben la diferenciación de los preadipocitos afectando sobre todo a la inducción de los factores de transcripción PPARγ y C/EBPα[152]. La **hormona de crecimiento** (GH, *Growth Hormone*) es otro factor de los que inhibe el proceso de adipogénesis y activa la lipolisis[153]. También las **citoquinas inflamatorias** como son TNFα (*Tumor Necrosis Factor alpha*), IL-1(*Interleukin 1*), IL-6, IL-11 o IFNγ (*Interferon gamma*) inhiben la diferenciación de los preadipocitos[154-156]. Del mismo modo **TGF-β** (*Transforming Growth Factor 1*) es capaz de estimular la proliferación de los preadipocitos inhibiendo su diferenciación[157].

3. B. IV. EL TEJIDO ADIPOSO BLANCO COMO ORGANO DE RESERVA Y MOVILIZACIÓN DE LIPIDOS

Durante la alimentación, los lípidos que provienen de la dieta son básicamente triglicéridos (TAG, *triacylglycerol*), algunos ácidos grasos libres (FFA, *Free Fatty Acids)* ,

colesterol y otros esteroles. Estos lípidos pueden acceder al torrente sanguíneo como quilomicrones, vía el sistema linfático o vía el hígado que ayuda a distribuir los lípidos, esterificando los ácidos grasos a triglicéridos y empaquetándolos en forma de VLDL (*Very Low Density Lipoprotein*). Una vez que los quilomicrones y las VLDL interaccionan con LPL (*Lipoprotein Lipase*) de las células endoteliales, se liberan ácidos grasos no esterificados (NEFA). Los ácidos grasos se transportan dentro de las células principalmente por dos tipos de transportadores: los CD36 y la familia de proteínas transportadoras de ácidos grasos (FATP, *Fatty Acid Transport Protein*) formada por 6 miembros no homólogos a CD36. Una vez en las células los ácidos grasos se esterifican a aciles CoA y su destino varía dependiendo del tipo celular. En el tejido adiposo de humanos cercanos a su peso ideal, el primer destino de los Aciles CoA de ácidos grasos es su reesterificación a triglicéridos[158].

En cambio durante el ayuno el tejido adiposo se convierte en fuente principal de ácidos grasos. Se da una caída del ratio insulina-glucagón y las catecolaminas aumentan activando la lipólisis en el tejido adiposo, hecho que genera ácidos grasos y glicerol. De este modo el ayuno provoca la estimulación de la oxidación de los ácidos grasos y un descenso del uso de la glucosa en varios tejidos para que otros tejidos que dependen de ella exclusivamente, como el cerebro, puedan seguir funcionando. El hígado es el primer órgano en el que se inhibe la oxidación de glucosa, reduciéndose el flujo de sus metabolitos hacia las vías mitocondriales. También se inhibe la lipogénesis y se estimula la oxidación de los ácidos grasos, básicamente por la des-represión de la CPT1 (*Carnitine Palmityl Transferase 1*), enzima que permite la entrada de ácidos grasos a la mitocondria. Esta oxidación de ácidos grasos conlleva la inhibición de la oxidación de la glucosa ya que la acumulación de Acetil CoA, NADH y ATP inhiben alostericamente la piruvato deshidrogenasa (PDH). El efecto neto de todo este proceso es un aumento de tasa de oxidación de ácidos grasos y una disminución de la glucosa[158]. Por otro lado también se observa durante el ayuno un descenso de la expresión del transportador de glucosa GLUT-4 en el tejido adiposo, asociado a cierta resistencia a la insulina[159].

3. B. V. EL TEJIDO ADIPOSO BLANCO COMO ORGANO ENDOCRINO

Tradicionalmente el tejido adiposo blanco ha sido considerado como un almacén de lípidos metabólicamente pasivo. En cambio en 1994, se descubrió la leptina, una hormona secretada por el tejido adiposo blanco, que revolucionó la idea que se tenía del tejido adiposo blanco. Actualmente está ampliamente aceptado que el tejido adiposo blanco es un órgano endocrino que secreta un alto número de hormonas y otros factores que pueden actuar a nivel autocrino o endocrino. A estas hormonas se les ha llamado adipoquinas y juegan un papel muy importante en el control de la ingesta, la homeostasis metabólica y en el desarrollo de alteraciones

metabólicas relacionadas con la obesidad, sobre todo la diabetes mellitus tipo II o las enfermedades cardiovasculares[160-162].

La primera adipoquina que se descubrió fue la hormona **leptina**. Es producida en un 95% por el tejido adiposo, especialmente por los depósitos subcutáneos. Sus niveles circulantes se correlacionan positivamente con el índice de masa corporal[163]. La falta de leptina o la de su receptor provocan obesidad mórbida tanto en ratones[164;165] como en humanos[166]. Sus niveles en plasma parecen estar afectados por la insulina, la entrada de glucosa a los adipocitos y por reguladores del sistema nervioso simpático[163;167]. En cuanto a sus funciones, actuando a nivel hipotalámico, la leptina inhibe la ingesta y activa el gasto energético y la función neuroendocrina, regulando de esta manera el peso corporal. Entre otros también se han descrito funciones reguladoras durante la pubertad y la reproducción, la función de la placenta, respuesta inmune y sensibilidad del musculo y el hígado a la insulina[168;169].

La **adiponectina** también es una adipoquina que se expresa preferentemente en el tejido adiposo blanco subcutáneo y circula por el torrente sanguíneo en forma de varias isoformas multiméricas[170]. Se conocen dos receptores de adiponectina, AdipoR1 y R2[171] que se expresan principalmente en el músculo y el hígado respectivamente. En pacientes obesos se ha observado un descenso de adiponectina en la obesidad[172], enfermedades relacionadas con la resistencia a insulina[173] o estados de inflamación[174], mientras que al recuperar un estado de peso normal se recuperan los niveles[173]. La administración de adiponectina recombinante revierte la resistencia a insulina incrementando la oxidación de ácidos grasos, la disipación de energía en el musculo esquelético, suprimiendo la inflamación en el propio tejido adiposo así como reduciendo la captación de glucosa por parte del hígado.

Los **ácidos grasos libres** no esterificados (NEFA) suponen el producto de secreción más importante de los adipocitos blancos, que también liberan otros derivados lipídicos como son el colesterol, retinol, hormonas esteroides y prostaglandinas[175]. Los NEFA provienen de la lipólisis que se da en los adipocitos y pasan al torrente sanguíneo donde normalmente aparecen acompañados por la albúmina. Además de su tarea como sustrato energético, también juegan un importante papel como moléculas señalizadoras relacionadas con la regulación de la expresión génica de diversos genes. Los tejidos lipogénicos como el hígado o el propio WAT son capaces de sintetizar *de novo* los ácidos grasos (lipogénesis) gracias a la glucosa, por ese motivo estos mismos tejidos son las principales dianas de regulación génica por parte de los ácidos grasos. De esta manera los NEFA son capaces de estimular la transcripción de proteínas implicadas en el transporte y el metabolismo de ácidos grasos, como son FABP (*Fatty Acid Binding Protein*), la LPL ,la CPT-1 o las proteínas desacoplantes 2 y 3, entre otras. Del mismo modo también son capaces de inhibir la transcripción de algunas proteínas implicadas en el metabolismo como son el

transportados GLUT4 o la L-PK (*Liver Piruvate Kinase*) así como la FAS (*Fatty Acid Synthase*)[176]. Uno de los mecanismo para llevar a cabo su función de reguladores de expresión génica es uniéndose de forma directa a algunos receptores nucleares de hormonas, como los PPAR[131].

Las **citoquinas proinflamatorias** son otro producto secretado por el tejido adiposo blanco. En 1993 se demostró que ratas obesas sufrían un aumento de expresión de TNFα en su tejido adiposo[177]. La TNFα es una proteína transmembrana que sufre procesos proteolíticos por los cuales se da lugar a la forma activa de la proteína que actúa sobre dos receptores (I y II). Su síntesis se da tanto en adipocitos como en células del estroma vascular[178]. En un principio se relacionó con la caquexia, pero hoy en día se sabe que está implicado en la patogénesis de la obesidad y la resistencia a insulina[177;179]. Desde que se descubriera la TNFα se han descrito una serie de factores pro-inflamatorios que van ganando protagonismo en la determinación de la obesidad como un estado inflamatorio siendo el tejido adiposo uno de los órganos patogénicos principales. Entre estas citoquinas pro-inflamatorias encontramos TGFβ, el interferón γ, interleuquinas (1, 6, 10 o 8), MCP1 (*Monocyte Chemotactic protein 1*) y otros factores de la cascada del complemento como pueden ser metalotioneína, fibrinógeno o plasminógeno[180-183]. Estas pueden ser producidas por células del estroma vascular y también por adipocitos. Cuando la masa del tejido adiposo aumenta estos factores circulantes aumentan, y relacionado a este aumento de masa también se ha descrito un aumento en la activación de dos cascadas típicamente inflamatorias como son la de la JNK (*c-Jun NH_2-terminal Kinase*)[184;185] y la de la IKKβ (*IkappaB Kinase β*)[186] en hepatocitos, hecho que se ha asociado a la resistencia a insulina.

La **proteína de unión a retinol** RBP4 (*Retinol Binding Protein 4*) es el transportador especifico de retinol (vitamina A), y a pesar de que el hígado es el órgano principal de secreción el tejido adiposo también es capaz de sintetizarla y secretarla en cantidades importantes que pueden llegar a significar el 20% de los producido por el hígado[187]. En 2005 se sugirió que el tejido adiposo secretaría RBP4 ante la ausencia de glucosa para inhibir la señalización por la insulina en el músculo y activar la expresión en hígado de PEPCK (*Phosphoenolpyruvate carboxykinase*), un enzima gluconeogénico, siendo el resultado neto un aumento de glucosa circulante[188]. Pero estudios posteriores han puesto en debate la relación de RBP4 con la resistencia a insulina.

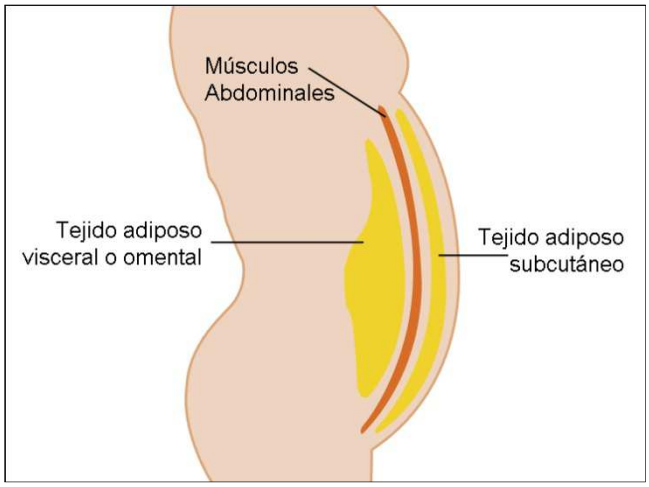
La **resistina** es otra adipocitoquina. Originariamente fue descrita como inductora de la resistencia a insulina[189;190]. La producción de resistina en humanos se da en diferentes tejidos entre ellos el tejido adiposo blanco. Parece que las células que más altamente la expresan son los PBMCs (*Peripheral-Blood Mononuclear cells*). Su expresión parece estar inducida por citoquinas proinflamatorias como las

interleuquinas 1 y 6 o el TNFα[191]. También se ha descrito que la administración de agonistas PPARγ atenúa su expresión[190;192]. Hoy en día se debate su implicación en la resistencia a insulina.

3. B. VI. DISTRIBUCIÓN DEL TEJIDO ADIPOSO BLANCO, EL DEPOSITO VISCERAL FRENTE AL SUBCUTANEO

El tejido adiposo blanco se distribuye anatómicamente en diferentes depósitos por el cuerpo de manera subcutánea en lo conocido como tejido adiposo subcutáneo (SAT, *Subcutaneous Adipose Tissue*) o en un depósito intra-abdominal llamado tejido adiposo visceral u omental (VAT, *Visceral Adipose Tissue*). Las áreas en las que principalmente podemos encontrar tejido adiposo blanco subcutáneo son las regiones femorogluteales y los depósitos abdominales. Entre este último y el tejido adiposo visceral se encuentran los músculos abdominales marcando una clara separación entre ambos. Esta distribución es importante ya que existen diferencias entre las características estructurales y funcionales entre el depósito visceral y el depósito subcutáneo. El 80% de la grasa total en humanos, se encuentra en el tejido adiposo subcutáneo, y el depósito visceral cuenta con un 10-20% en hombres y un 5-8% en mujeres[193;194].

Figura 12. Distribución anatómica diferencial de los depósitos de tejido adiposo visceral y subcutáneo.



La acumulación de grasa en forma de triglicéridos en el tejido adiposo subcutáneo representa el almacenaje habitual del exceso de energía obtenido en la dieta. Pero en algunas situaciones en las cuales se excede la capacidad de almacenaje de este depósito o existe algún motivo que inhibe la capacidad de generar nuevos adipocitos en el tejido adiposo subcutáneo, bien sea por una predisposición genética o por motivo del stress, el exceso de energía comienza a acumularse en otras áreas

como el tejido adiposo visceral[195]. A pesar de esto, es difícil establecer un patrón por el cual el tejido adiposo visceral aumenta de volumen ya que, a pesar de su relación con el aumento de BMI (*Body Mass Index*) y obesidad, existen individuos no obesos que presentan un aumento de adiposidad visceral en los cuales se aprecian diferencias metabólicas inter-individuales y del mismo modo, pacientes obesos que no muestran un aumento del depósito omental, indicando que puede existir una predisposición de algunos individuos a acumular grasa en este depósito omental[193;196].

Observando la distribución de los depósitos omental y subcutáneo, una de las primeras diferencias que podemos apreciar es que el depósito visceral está directamente irrigado por la vena porta[197], en cambio el tejido adiposo subcutáneo aparece irrigado por venas de la circulación sistémica periférica. La vena porta, conduce directamente al hígado y debido a esto los FFA y las adipoquinas secretadas por el tejido adiposo visceral tendrán una conexión inmediata con el hígado pudiendo generar diferentes respuestas en éste[198;199]. Además en este ámbito, cabe destacar que el tejido adiposo visceral está más vascularizado, más irrigado y más inervado que el subcutáneo[198].

En cuanto a los adipocitos presentes en ambos tejidos adiposos se han observado algunas diferencias. Entre ellas es interesante el hecho de que el tejido adiposo visceral presente un mayor tamaño medio de adipocitos en contraste con el subcutáneo, que si bien posee adipocitos de gran tamaño, también se pueden observar pequeños adipocitos[197;200;201]. Este hecho, que ha sido observado significativamente en un estudio con hombres, está relacionado con una tendencia mayor a la resistencia a la insulina en el tejido adiposo visceral, ya que los adipocitos de mayor tamaño resultan más disfuncionales mostrando esta resistencia al efecto anti-lipolítico de la insulina y siendo metabólicamente más hiper-lipolíticos que los adipocitos presentes en el SAT[197;200;201]. En otros estudios se ha demostrado que los adipocitos del VAT son más insulino resistentes o resistentes a la acción anti-lipolítica de la insulina que aquellos del SAT[99;202-204], y esto parece estar altamente relacionado con las pruebas que muestran que los pacientes con mayor volumen de grasa en el depósito omental sufren una mayor resistencia a la insulina a pesar de existir un debate sobre si es una causa o un efecto[204]. De acuerdo con esto, diferentes estudios han determinado que los adipocitos de SAT presentan una mayor captación de triglicéridos y FFA que los viscerales y en cambio parece que los adipocitos de la zona visceral son capaces de captar más glucosa[198;201].

Resulta interesante la diferencia en la presencia de diferentes receptores implicados en las funciones del tejido adiposo que se evidencia entre ambos depósitos y que además pueden explicar las diferencias inter-sexuales que se muestran entre los tejidos adiposos subcutáneos y el omental. Los receptores de andrógenos presentan, una mayor densidad en el VAT que en el SAT[205]. En cambio la densidad de receptores

de estrógenos parece ser mayor en el tejido adiposo subcutáneo[206], hecho que podría ser protectivo contra la acumulación de grasa en el VAT, ya que favorecería la acumulación periférica de la grasa. Este hecho va acorde con las diferencias inter-sexuales que se aprecian en cuanto a la adiposidad visceral. Con la edad en ambos sexos el tejido adiposo subcutáneo va descendiendo de volumen mientras el tejido adiposo visceral va aumentando la acumulación de grasa, pero este hecho es más patente en hombres que en mujeres[207], ya que los estrógenos parecen tener una función protectora en estas últimas. En cambio en mujeres post-menopáusicas la curva de aumento de la adiposidad visceral se iguala a la de los hombres[207]. De esta manera, siendo los andrógenos movilizadores de lípidos, y reduciéndose en hombres los niveles de testosterona con la edad, y reduciéndose los estrógenos en las mujeres tras la menopausia, adquiere sentido la correlación existente entre la acumulación de grasa en el VAT y la edad.

En cuanto al control nervioso de la lipólisis, se ha descrito un aumento en la cantidad de receptores β_3-adrenérgicos en el VAT respecto al SAT. Estos receptores han sido descritos como capaces de provocar lipólisis vía aumento de cAMP en adipocitos aislados de tejido adiposo visceral[208;209]. Este hecho se suma a los estudios que indican que el tejido adiposo visceral es más sensible que el subcutáneo a la lipólisis provocada por las catecolaminas por una mayor sensibilidad de sus receptores β adrenérgicos 1 y 2 que los mismos en el SAT[210]. Además también se ha observado una menor sensibilidad en el tejido adiposo visceral de los receptores inhibidores de la lipólisis α_2-adrenérgicos, hecho que refuerza el estado metabólico más lipolítico debido a la mayor sensibilidad a las catecolaminas de este depósito omental[211;212].

También se han descrito diferencias entre el VAT y el SAT en cuanto a la síntesis y la secreción diferencial de adipoquinas en ambos depósitos. El tejido adiposo subcutáneo parece secretar más leptina[213;214], siendo la mayor fuente de esta. En cambio la adiponectina se expresa en mayor cantidad en el VAT[215]. En cuanto a las adipoquinas proinflamatorias como la IL6 o IL8, es interesante comentar que el tejido adiposo visceral está más infiltrado por células inflamatorias que el SAT, y por lo tanto presenta una mayor capacidad de generar estas proteínas que este ultimo[216;217]. Es interesante mencionar en este ámbito, que el VAT se asocia también con una mayor secreción de MCP1[218].

El aumento de adiposidad visceral se ha relacionado estrechamente con la resistencia a la insulina y el síndrome metabólico[193;219]. De tal manera que hoy se ha convertido en una de las alteraciones que forman parte de la combinación de disfunciones que forman dicho síndrome como son: hipertrigliceridemia, hipercolesterolemia, resistencia a insulina, hipertensión y la propia adiposidad visceral que suele ser medida por el WHR (*Waist to Hip Ratio*) o técnicas de imagen como la DEXA (*Dual-emission X-ray absorptiometry*). De hecho es uno de los síntomas que

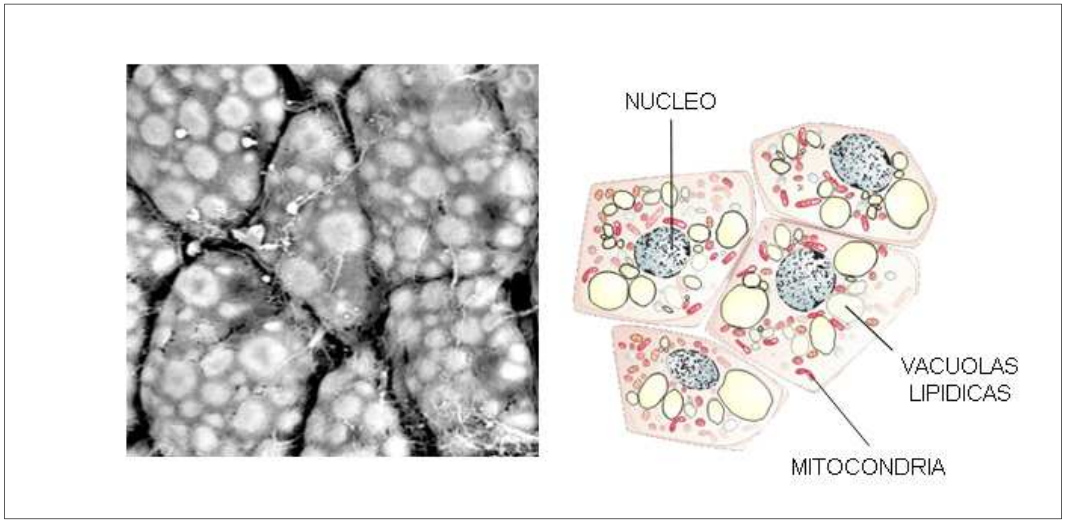
sugieren una futura aparición del síndrome metabólico[193]. De este modo existen un gran número de estudios que asocian la adiposidad visceral y no la subcutánea con un alto riesgo de sufrir accidentes cardiovasculares[220]. Este hecho tiene sentido asumiendo que existe una hiperinsulinemia asociada a la adiposidad visceral que puede provocar una hipertensión arterial y un aumento en la secreción de PAI1, factor trombogénico, por parte de este tejido adiposo[221].

La capacidad del tejido adiposo visceral de ser metabólicamente más lipolítico que el subcutáneo por su mayor sensibilidad a las catecolaminas y además siendo su respuesta a la insulina más resistente que en el depósito subcutáneo, parece indicar que este tejido adiposo omental contribuye en mayor medida al aumento de FFA en plasma[193;194]. Como se ha comentado la irrigación por la porta de este tejido adiposo omental provoca una conexión directa con el hígado y de esta manera, diversos autores han propuesto que se podría dar un aumento crónico de FFA en plasma provocado por este hecho y que esto podría provocar disfunciones hepáticas provocando finalmente una intolerancia a glucosa, hiperinsulinemia, resistencia a insulina o dislipidemia, explicando la relación entre el aumento en el tejido adiposo visceral y la resistencia a la insulina y el síndrome metabólico[194].

3. C. TEJIDO ADIPOSO MARRÓN

En los últimos años se está llevando a cabo un (re)descubrimiento del tejido adiposo marrón en humanos. Se asumía que este tejido, importante para los neonatos humanos desaparecía a los pocos meses del nacimiento. Fue descrito que algunas células adiposas del tejido adiposo blanco, eran capaces de expresar marcadores específicos de tejido adiposo marrón, como la proteína UCP1 (*Uncoupling Protein 1*) asumiéndose que algunos adipocitos del tejido adiposo blanco tenían un fenotipo *brown-like* o que existían adipocitos marrones infiltrados en el tejido adiposo blanco. Pero fue más tarde y gracias al PET (Positron Emission Tomography) cuando se pudo identificar los depósitos de tejido adiposo marrón en humanos adultos[222]. Hoy en día se asume la presencia de este tejido adiposo en humanos y se relaciona su presencia y funcionalidad tanto con la termorregulación, como se explica a continuación como con un gasto energético que ayuda a mantener la homeostasis metabólica evitando un excesivo almacenaje de grasas, habiéndose convertido el tejido adiposo marrón en una nueva diana para presentes y futuras investigaciones que intentan evitar la cada día más común entre las sociedades desarrolladas obesidad y sus problemas metabólicos asociados[223;224].

Figura 13. Morfología típica de los adipocitos marrones.



La capacidad de mantener la temperatura corporal o eutermia es una de las funciones básicas de los animales superiores. Existen diversos mecanismos dirigidos a generar calor y mantener la temperatura. En cuanto a la generación de calor se destacan dos mecanismos. Uno implica, en aves y mamíferos el temblor provocado por el sistema muscular que se contrae involuntariamente para producir calor. El otro, en mamíferos, es la termogénesis no asociada a temblor que se lleva a cabo en el tejido adiposo marrón[225]. Por otro lado este mismo tejido juega un importante papel en la termogénesis adaptativa inducida por la dieta que sirve para regular el gasto energético y el peso corporal.

El tejido adiposo marrón se localiza en los pequeños mamíferos en diversas zonas del organismo como la región interescapular, cervical, axilar, intercostal y envolviendo la aorta, el timo y los riñones[226]. Esta distribución permite la transferencia de calor a los órganos y vasos sanguíneos principales. En humanos se aprecia una distribución, además de la forma difusa en la que se mezclan adipocitos marrones en el tejido adiposo blanco, que implica zonas cervicales-supraclaviculares así como renales o perirenales y zonas paravertebrales cercanas a los vasos principales[222].

El color marronoso del tejido adiposo marrón viene dado por su alta vascularización así como por su alto contenido en mitocondrias. La alta vascularización responde a una demanda de una alta tasa de perfusión para llevar a cabo la actividad termogénica, ya que esta requiere mucho oxigeno y sustratos que necesarios para las mitocondrias, así como un medio para exportar el calor producido[227]. Es importante también en este tejido la alta inervación del sistema nervioso simpático que la controla desde tres áreas del cerebro: El hipotálamo anterior, que controlaría la actividad del tejido al bajar la temperatura, el centro hipotalámico ventromedial (centro de la

saciedad) que activa el tejido en relación a la ingesta y por último, el centro lateral hipotalámico (centro del hambre) que inhibe la actividad del tejido durante el ayuno.

En el tejido adiposo marrón hay principalmente adipocitos marrones que representan un 40% del total de las células de este tejido[228]. El resto son células endoteliales, fibroblastos, células perivasculares mesenquimales, preadipocitos, mastocitos y células de Schwan que conforman el estroma vascular. El sustrato principal de los adipocitos marrones son los ácidos grasos que se almacenan en forma de TAG en las numerosas gotas lipídicas que podemos encontrar en su citoplasma. Cuanto mayor es la actividad de la célula más pronunciada es la disposición multivacuolar de los depósitos de lípidos, lo que permite una más fácil movilización de las reservas para su oxidación. Esta oxidación se lleva a cabo en las numerosas mitocondrias, grandes, de morfología alargada y con un alto número de crestas altamente desarrolladas que indican su alta actividad. La membrana de estos adipocitos presenta un alto número de receptores α y β adrenérgicos[226].

La capacidad de producir calor que presenta este tejido es debida a un desacoplamiento regulado de la cadena respiratoria y la fosforilación oxidativa que permite que la energía se disipe en forma de calor. Un adipocito marrón diferenciado expresa en sus mitocondrias UCP1, que es la proteína de la membrana mitocondrial interna que permite este desacoplamiento y la comentada producción de calor[226].

3. C. I. DIFERENCIACIÓN ADIPOCITARIA DEL TEJIDO ADIPOSO MARRON

Los adipocitos marrones, como los blancos derivan de las células del mesodermo[229]. Su proceso de adipogénesis no esta tan bien caracterizado como el del adipocito blanco. Su desarrollo en mamíferos se da sobre todo durante el periodo fetal o inmediatamente post-natal madurando los primeros días de vida[226]. A lo largo de su diferenciación intervienen también factores de transcripción de la familia de los PPAR y de los C/EBP. También adquiere una gran importancia en la diferenciación del adipocito marrón el coactivador PGC-1α (*PPARγ Coactivator 1*)[230] y el recientemente descubierto PRDM16[231].

A los largo de su diferenciación adquieren la típica morfología multivacuolar y con un alto número de mitocondrias, así como la capacidad específica termogénica con la expresión de genes característicos del metabolismo lipídico y de la biogénesis y diferenciación mitocondrial, así como la expresión de UCP1, considerado el principal marcador de este tejido[226].

3. C. II. CASCADA DE ACTIVACION DE LA DIFERENCIACION Y FACTORES IMPLICADOS

Tal y como sucede en el adipocito blanco, los factores de transcripción que son considerados responsables del proceso de diferenciación de los adipocitos marrones pertenecen básicamente a las familias PPAR y C/EBP.

PPARγ presenta un perfil de expresión parecido a C/EBPα y su papel podría ser similar al que tiene en el tejido adiposo blanco[232;233]. La administración de TZD promueve igualmente la diferenciación de los preadipocitos e induce el crecimiento del tejido adiposo interescapular[234]. Siguiendo con la familia de los PPAR, se cree que PPARδ controla la proliferación de preadipocitos[235]. PPARα parece estar inducido por la disminución de expresión de PPARδ[236], y a diferencia del tejido adiposo blanco su expresión es alta y tiene lugar posteriormente a la de PPARγ y PPARδ, coincidiendo con la adquisición del fenotipo diferenciado terminal. En el tejido adiposo marrón los PPAR inducen la expresión de UCP1 y UCP3, proteínas marcadoras de la adquisición del fenotipo marrón diferenciado[232]. Entre los diferentes estudios realizados en tejido adiposo marrón y blanco se intuye que PPARγ no es, aparentemente, el primer factor en determinar si una célula adiposa asume un fenotipo marron[237;238], de hecho ambos tipos de adipocitos parecen estar determinados a un tipo de diferenciación dependiendo de su origen fibroblastico[239;240] (figura 14).

La importante implicación del tejido adiposo marrón en el balance energético ha motivado un gran interés por conocer las claves de la determinación de la diferenciación de estos adipocitos ya que se busca el modo de transformar células adipocíticas blancas en marrones para convertir células que almacenan grasa en células que las oxidan. En este aspecto es importante destacar PGC1α, coactivador con una importante presencia en el tejido adiposo marrón respecto al blanco. Este coactivador es capaz de activar el programa termogénico cuando es expresado ectópicamente en adipocitos blancos, haciendo que expresen UCP1, proteínas de la cadena respiratoria y enzimas que toman parte en la β-oxidación mitocondrial[241;242]. Este coactivador está implicado en múltiples procesos relacionados con el metabolismo energético celular y la capacidad y funcionamiento mitocondrial. Preadipocitos de TAM carentes de PGC1α son capaces de diferenciarse, pero en cambio se reduce severamente la inducción de genes termogénicos vía cAMP[243]. Por tanto PGC1α parece esencial para la respuesta termogénica pero no para la diferenciación del adipocito marrón[243].

Otro factor que parece influenciar selectivamente el fenotipo marrón es FOXC2 (*Forkhead box C2*), que es capaz de inducir algunas características de adipocitos marrones en blancos[244]. Es interesante resaltar que se han descrito factores que reprimen la expresión del fenotipo marrón en adipocitos blancos, como son: Rb (*retinoblastoma*), p107 o RIP140 (*Receptor Interacting Protein 140*)[245-247].

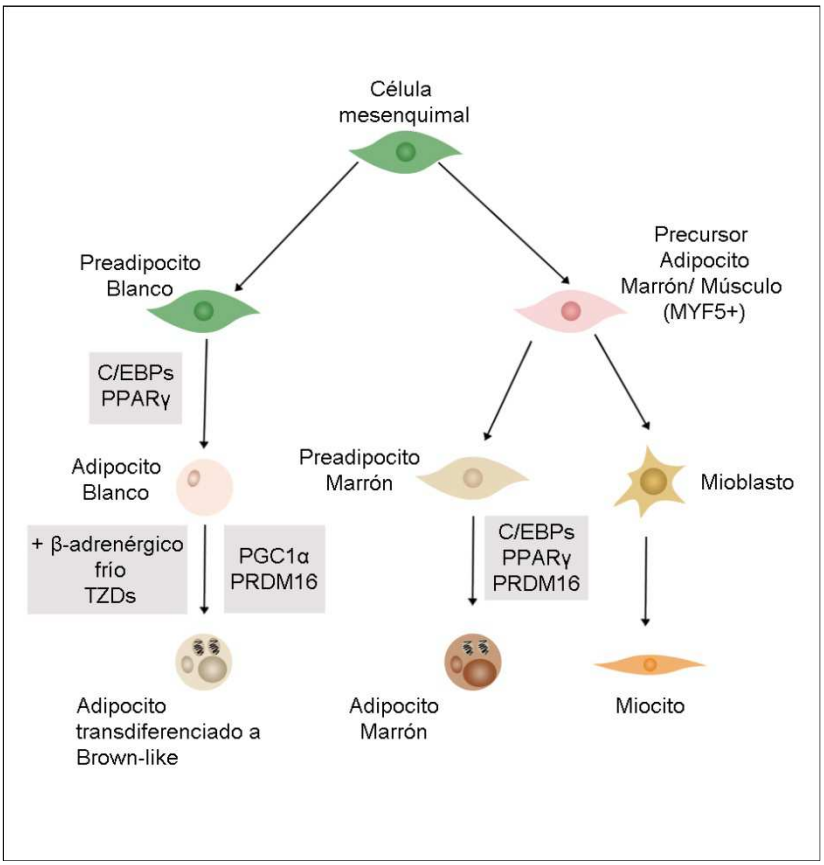
Recientemente se ha identificado PRDM16 (*PRD1-BF1-RIZ1 homologous domain-containing protein 16*) o MEL1 como un factor clave en la diferenciación adipocitaria marrón[231]. Se trata de una proteína que se expresa en el hígado, riñón y cerebro y de forma diferencial en el tejido adiposo marrón respecto al blanco. Se expresa en adipocitos maduros. No se aprecia que su expresión se induzca por el frío o por la presencia de cAMP, lo que sugiere que está más relacionada con la determinación y la diferenciación que con la respuesta termogénica. Su expresión ectópica en tejido adiposo blanco es capaz de inducir la expresión de genes característicos del BAT como UCP1, PPARα, la desiodasa 2(DIO2), PGC1α y genes del sistema OXPHOS (*oxidative phosphorilation*), así como de reprimir genes específicos del tejido adiposo blanco como la resistina y la adiponectina[231].

La ausencia de PRDM16 en cultivos primarios de adipocitos marrones promueve una diferenciación hacia células musculares y lo mismo ocurre con ratones deficientes en PRDM16. Esto indica que PRDM16 es un determinante crítico para la determinación del linaje del precursor de tejido adiposo marrón[231].

Parece que PRDM16 actúa de manera independiente a unión a DNA. Este hecho sugiere que su acción tiene lugar a través de la interacción con otras proteínas. Se ha descrito que PRDM16 forma un complejo transcripcional con C/EBPβ[248]. De hecho la depleción de C/EBPβ impide la acción de PRDM16 de inducir la diferenciación a adipocito marrón y la combinación de estos dos factores es suficiente para inducir un fenotipo marrón en células no adipogénicas como fibroblastos embrionarios o de la piel.

Estas evidencias y otras acumuladas a lo largo de los últimos años parecen indicar que los adipocitos marrones tienen un origen más cercano a los miocitos que a las células del tejido adiposo blanco. Estudios de caracterización indican que los adipocitos marrones de la zona interescapular y el músculo esquelético, a diferencia de los adipocitos blancos, provienen de las células que expresan Myf5, un gen que hasta ahora se asumía que se expresaba exclusivamente en células de linaje muscular[249]. Además se ha descrito en estudios de expresión génica global que los precursores de adipocitos marrones muestran un perfil de expresión muy parecido a las células de músculo esquelético, mientras que los precursores del tejido adiposo blanco no. También se ha determinado que el perfil proteómico del BAT es más cercano con el perfil muscular que con el de WAT[250].

Figura 14. Relación entre la diferenciación adipogénica de los adipocitos marrones y los blancos con los factores implicados con una posible vía de transdiferenciación de los adipocitos blancos a un fenotipo Brown-like por diversos estímulos. (Adaptado de Cristancho A. *et al*. *Nature*. 2011)



Uno de los factores que se cree que puede estar relacionado con la expresión de PRDM16 y PGC1α en adipocitos y fibroblastos multipotenciales es BMP7 (*Bone Morphogenetic Protein-7*), miembro de la superfamilia TGFβ[251].

Como se ha comentado existen células adiposas marrones dentro del WAT. Estos adipocitos marrones expresan UCP1 y receptores β-3 adrenérgicos pero no parecen tener un origen común a los adipocitos marrones del BAT ya que sus precursores no expresan Myf5. Hasta el momento no se ha podido determinar cómo estos adipocitos adquieren un fenotipo *brown-like* y si tienen un origen común al de los adipocitos blancos[249].

3. C. III. TERMOGENESIS ADAPTATIVA Y FACTORES QUE LA REGULAN

En general la capacidad del tejido adiposo marrón de generar calor depende de la temperatura ambiental, el estado de desarrollo y de la ingesta, ya que estos factores parecen influir en la cantidad de proteína desacopladora presente en la membrana de la mitocondria como su actividad. El principal estímulo para la termogénesis es la disminución de la temperatura ambiental que se asocia a un aumento en la expresión de UCP1[252]. En la exposición aguda al frio se observa un incremento de actividad simpática, una rápida respuesta termogénica en el tejido adiposo marrón y un aumento de flujo sanguíneo en este tejido. En una exposición crónica aumenta la proliferación y diferenciación de preadipocitos (*recruitment)*, proliferación de mitocondrias e incremento de síntesis de proteínas UCP1, LPL, DIO2 y enzimas implicados en la β oxidación[253].

Durante la ingesta también se estimula el tejido adiposo marrón, incrementándose la actividad del sistema nervioso simpático sobre el tejido adiposo marrón, estimulando la actividad termogénica[254]. De esta manera el BAT participa en la regulación del peso corporal[255]. Los animales que son sometidos a dietas hipercalóricas, presentan una hipertrofia del tejido adiposo marrón y una alta expresión de UCP1[256].

Además, hormonas como la leptina[257] o las hormonas tiroideas[258;259] también regulan positivamente la actividad del BAT. Y por otro lado se disminuye su actividad por altas temperaturas, durante el ayuno[260], dietas hipocalóricas[261] o por glucocorticoides[262].

La noradrenalina (NA) es capaz de interactuar con diferentes receptores adrenérgicos que se asocian a diferentes vías de señalización. El BAT expresa los 3 receptores β adrenérgicos (1, 2 y 3), siendo el β1 y el β3 los que participan en la respuesta termogénica[263]. Estos receptores están acoplados a proteína G[264] y cuando son estimulados se incrementan los niveles de cAMP a través de la adenilato ciclasa. Este aumento activa PKA (*Protein Kinase A*) que es capaz de activar la lipasa sensible a hormonas (HSL, *Hormone Sensitive Lipase*) que incrementa la lipólisis y promueve consecuentemente la termogénesis favoreciendo la activación de UCP1 por los ácidos grasos liberados[265]. Además a la vez que esto sucede se da una inducción de expresión de UCP1. PKA activa una cascada de proteínas quinasas (*como la MKK3 Map Kinase Kinase 3)* que finaliza con la activación de p38 MAPK (*Mitogen Activated Protein Kinase*)[266]. La p38 es capaz de fosforilar el factor de transcripción ATF2 (*Activating Transcription Factor 2*) y CREB (*cAMP Response Element Binding protein*). Además PKA contribuye de forma directa también a la fosforilación de CREB. Por último estos factores se unen a elementos de respuesta en el promotor de UCP1 y PGC1α activando su transcripción[267;268]. Por otro lado la p38 también fosforila PGC1α que coactiva otros

factores de transcripción que se unen al promotor de UCP1 como los PPAR, RAR (*Retinoic Acid Receptor*) y TR (*Thyroid hormone Receptor*).

Todos estos procesos permiten mantener la respuesta termogénica aumentando la mitocondriogénesis dependiente de PGC1α y aumentando los niveles de proteína UCP1[269;270]. Los receptores β1-adrenérgicos están involucrados en el incremento de la división celular que se da en el BAT en respuesta al frio[271].

El BAT también presenta dos subtipos de receptores α-adrenérgicos (1 y 2). La activación de los α_2 estimula la proteína Gi e inhibe la adenilato ciclasa disminuyendo los niveles de cAMP que se producen durante la estimulación simpática del tejido. Los α1 en cambio producen un aumento de inositol-trifosfato[272] y un aumento de calcio intracelular[273]. La estimulación simultanea de los receptores α1 y los β-adrenérgicos resulta en un incremento del efecto provocado por el cAMP[272].

El tejido adiposo marrón es capaz de producir hormona tiroidea activa (T3, 3,3´5-triiodotironina) a partir de tiroxina (T4) gracias a la expresión en el BAT del enzima desiodasa 2 (*type II iodothyronine deiodinase*)[259] cosa que no ocurre en el WAT. La T3 producida en el BAT puede incluso ser liberada al torrente sanguíneo[274]. La T3 es necesaria para la termogénesis inducida por el frio y actúa de forma sinérgica con la noradrenalina a diferentes niveles: activando la adenilato ciclasa, en el efecto de cAMP sobre la lipólisis y en la acción termogénica de los ácidos grasos sobre las mitocondrias[275]. El efecto del frio sobre la acción de la desiodasa 2 viene vehiculado por los receptores α1 y β-adrenérgicos[276]. La hormona que se produce en situaciones de frio es suficiente para saturar sus receptores nucleares y activar junto a la NA, la expresión de UCP1[259;277] al unirse a sus elementos de respuesta en el promotor[278;279].

Otro factor a tener en cuenta es el ácido retinoico (RA, *retinoic acid*) derivado de la vitamina A que toma parte en la proliferación celular, morfogénesis y diferenciación de diversos tejidos en mamíferos. Hay dos isómeros que principalmente llevan a cabo esta función, se trata de *9-cis* RA y el *all-trans* RA. Su función se lleva a cabo a través de factores de transcripción dependientes de ligando como son el RAR y el RXR, siendo el *all-trans* RA ligando específico del RAR y el *9-cis* RA panagonista de ambos factores de transcripción[280]. Los efectos sobre RAR en adipocitos marrones activan la expresión de UCP1[281;282] por una parte y por otra, paradójicamente, pueden inhibir la diferenciación a adipocito, cosa que sucede también en los adipocitos blancos[283]. Por otra lado el ácido retinoico *9-cis*, capaz de activar RXR tiene efecto positivo sobre la diferenciación de los adipocitos marrones, tal y como sucedía con los blancos[284-286].

La insulina contribuye al mantenimiento de la capacidad termogénica del BAT y participa en el metabolismo del adipocito marrón estimulando la captación de glucosa y ácidos grasos, así como su oxidación. Se ha descrito que interviene en la diferenciación a adipocitos marrones uniéndose a sus receptores de insulina/IGF1 y

activándolos, hecho que suprime la expresión de necdina, una proteína que controla señales represoras de la diferenciación[287]. El IGF1 se expresa en el tejido adiposo marrón al final del desarrollo fetal, momento en el que se da la diferenciación adipocitaria y termogénica y parece estar implicado en la diferenciación adipocitaria y en la expresión, y su mantenimiento, de la proteína UCP1[288].

Estudios recientes llevados a cabo en nuestro laboratorio han identificado FGF21 (*Fibroblast Growth Factor 21*) (ver apartado 5. C.) como un factor activador de la termogénesis en el tejido adiposo marrón[289]. FGF21 es miembro de la superfamilia FGF y recientemente ha surgido como un regulador importante del metabolismo[290]. La principal fuente de FGF21 es el hígado desde donde llega al BAT para activar la termogénesis a través del aumento de expresión de genes implicados en este proceso como PGC1α, UCP1, el citocromo c o GLUT4[289]. De este modo estudios recientes de nuestro laboratorio indican que el propio BAT produce FGF21 que gracias a sus efectos autocrinos aumentan, del mismo modo, la actividad termogénica del tejido[291].

Pero si existe un factor que juegue un papel fundamental en la modulación de la expresión de genes termogénicos, es PGC1α. En ratones expuestos al frio la expresión de PGC1α en el BAT se induce considerablemente y también se observa inducción en adipocitos marrones en cultivo tratados con isoproterenol (agonista β3-adrenérgico)[241]. Se ha descrito que esta inducción está vehiculada por los receptores β3-adrenérgicos ya que animales deficientes en este receptor no sufren la típica inducción de expresión de PGC1α por el frio[292]. También se ha observado que el tratamiento con agonista β3-adrenérgicos promueve la inducción de PGC1α en el BAT[293]. Los adipocitos que no expresan PGC1α, son capaces de diferenciarse normalmente, pero presentan una inducción disminuida de genes termogénicos, hecho que sugiere que PGC1α es importante para la función termogénica, pero no afecta a otras facetas del adipocito como la diferenciación o la acumulación de lípidos[243].

Un factor importante en la termogénesis adaptativa es la biogénesis mitocondrial. PGC1α está relacionado con el control de esta biogénesis. La exposición al frio induce la proliferación de mitocondrias así como el desacoplamiento de la respiración. NRF1 y NRF2 (*Nuclear Respiratory Factor 1 and 2*) son dos factores de transcripción que regulan un alto número de genes mitocondriales codificados por el DNA nuclear[294]. Uno de estos genes es TFAM (*mitocondrial transcription factor A*) esencial para la replicación, mantenimiento y transcripción del DNA mitocondrial[295;296]. PGC1α interactúa físicamente con NRF1 aumentando su actividad transcripcional sobre sus genes mitocondriales diana entre los que está, como se ha comentado TFAM[297].

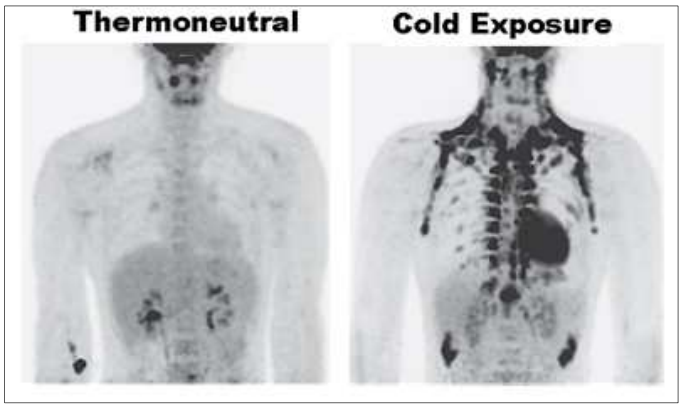
Por otro lado PGC1α también induce la expresión de enzimas que participan en la β-oxidación como son la MCAD (*Medium chain acyl CoA Dehydrogenase*) y la CPT1 a través de la coactivación de factores de transcripción como PPARα y ERRα (*Estrogen*

Related Receptor)[298]. De esta manera PGC1α es indispensable en el tejido adiposo marrón para establecer y coordinar una red de regulación transcripcional implicada en la disipación de energía tan característica de este tejido.

3. C. IV. TERMOGENESIS EN HUMANOS

La detección por PET de tejido adiposo marrón en humanos adultos ha sido un paso definitivo en el (re)descubrimiento de este tejido en el ser humano. El PET (*Positron Emission Tomography*) es una técnica utilizada para detectar células y tejidos cancerosos basándose en su capacidad de captar grandes cantidades de glucosa marcada. Durante estas pruebas se observaba la aparición de puntos de alta captación de glucosa distribuidos simétricamente por el cuerpo y cuya densidad coincidía con la del tejido adiposo y que variaban en función a la temperatura ambiente[299;300]. Estos datos fueron pasados por alto por expertos en metabolismo debido a su presencia solo en revistas muy especializadas, pero posteriormente han sido propuestas como prueba de la existencia de tejido adiposo marrón en humanos[301]. A esto se han sumado estudios de expresión génica en biopsias, revelando efectivamente la presencia de este tejido, que se pensaba desparecía a los pocos meses de nacer, en humanos adultos.

Figura-15. Imagen obtenida por PET de un individuo a temperatura termoneutral (izquierda) y expuesto a baja temperatura(derecha) en la que se aprecia, en negro, las zonas de tejido adiposo marrón reconocidas por ser metabólicamente más activas.(Adaptado de Marken Lichtenbelt et al. 2009)



La distribución del tejido adiposo marrón humano es básicamente supraclavicular, cervical, paravertebral, mediastinal, paraaórtica y suprarrenal. En estos depósitos se da una expresión de genes adiposos característicamente marrones como son UCP1, PGC1α o PRDM16. También se ha observado que su actividad aumenta notablemente en respuesta al frio[223].

Estos depósitos abren una puerta al estudio de la función de este tejido en la homeostasis del metabolismo energético y la posibilidad de su utilización como diana terapéutica en trastornos metabólicos o el balance energético[223].

Se debe tener en cuenta que en modelos animales la ausencia o la disfunción del BAT implica una reducción del gasto energético que finalmente suele derivar en la aparición de obesidad, así como de una resistencia a insulina. En humanos se han asociado polimorfismos de UCP1 o de los receptores β-adrenérgicos, a la obesidad y a la diabetes tipo II[302]. Estos hechos sugieren una relación entre tejido adiposo marrón, tasa metabólica y la obesidad con sus problemas asociados. A nivel de resistencia a insulina, cabe comentar que el BAT presenta una elevada oxidación de glucosa y una elevada expresión de GLUT4[288]. Este hecho y diferentes experimentos con animales indican que es un tejido muy importante para homeostasis de glucosa. Por eso cada día hay más interés en este tejido como diana terapéutica. El objetivo final de esta terapia consistiría en un aumento de la actividad de este tejido o de las células con fenotipo *brown-like* presentes en el tejido adiposo blanco, de manera que se diera un mayor gasto energético[303]. Hoy en día se buscan fármacos capaces de incrementar la actividad de este tejido y por otra parte se investiga en la diferenciación *exvivo* de precursores de estos adipocitos marrones de manera que pudieran ser reintroducidos en individuos obesos adultos[303].

3. C. V. EL TEJIDO ADIPOSO MARRÓN COMO ÓRGANO ENDOCRINO

Del mismo modo que sucedía con el tejido adiposo blanco, el BAT también es capaz de secretar una serie de moléculas y actuar como órgano tanto endocrino como paracrino.

A nivel de la **función paracrina** podemos destacar las siguientes moleculas.

La **adipsina** (factor del complemento D) es una serin proteasa sintetizada y secretada por los adipocitos marrones[304]. Los agonistas β3-adrenérgicos son capaces de inhibirla y normalmente se asocia negativamente con la activación del tejido adiposo marrón[304]. Su función podría ser la de funcionar como señal de un BAT termogénicamente inactivo en situaciones anabólicas.

El **NGF** (Nerve Growth Factor) es una neutrofina esencial para la supervivencia y el mantenimiento de las neuronas simpaticas[305]. Durante el periodo perinatal su secreción es elevada en le BAT principalmente por los preadipocitos. Este hecho promovería el desarrollo de la inervación simpática del tejido[306].

El **oxido nítrico** (NO) tiene una acción vasodilatadora muy potente. Los adipocitos marrones pueden producirlo cuando son estimulados con noradrenalina[307]. Este hecho parece estar relacionado con el incremento rápido y masivo de flujo sanguíneo al BAT cuando incrementa su actividad[308].

A un nivel de **función endocrina** se destacan las siguientes adipoquinas del tejido adiposo marrón.

Del mismo modo que en los adipocitos blancos, en los marrones también se produce **leptina** pero en este caso solo bajo condiciones de atrofia e inactividad. La actividad del BAT, en situaciones como el frio, se reduce su expresión[226].

Por otra parte cuando los adipocitos marrones son altamente estimulados por la noradrenalina y producen más **ácidos grasos** de los que pueden oxidar, estos son secretados[309]. Se desconoce si esta secreción tiene relevancia fisiológica o simplemente revela un balance entre la capacidad termogénica y la lipolítica.

Estos adipocitos marrones también expresan **adiponectina** y **resistina**. La regulación de la secreción de la resistina está poco clara y en cuanto a la secreción de adiponectina esta aparece disminuida cuando se da una estimulación adrenérgica en el adipocito marrón[226].

El tejido adiposo marrón es capaz de generar **T3** con función autocrina y endocrina. Cuando se da una estimulación adrenérgica por el frío su expresión y secreción aumentan , mientras disminuyen en el ayuno[274]. También se ha descrito que la T3 regula directamente la expresión de UCP1.

Como ocurre con el tejido adiposo blanco el tejido adiposo marrón tiene capacidad para producir y secretar **interleuquinas** como la IL1 y la IL6. Su expresión se estimula por LPS (Lipopolisacarido), IL-1β, TNFα o la noradrenalina[310;311].

4. RELACIÓN DEL HIV Y LA HAART CON EL TEJIDO ADIPOSO, EL SINDROME DE LIPODISTROFIA ASOCIADO A TERAPIA HAART

4. A. INTRODUCCIÓN

Como se ha comentado anteriormente, la introducción de la terapia HAART, logró un rápido descenso en la mortalidad y la morbilidad de los pacientes infectados por el virus HIV. A pesar de su éxito, pronto comenzaron a aparecer los efectos secundarios derivados de la combinación de los diferentes y potentes fármacos utilizados. Uno de los efectos secundarios más frecuente entre los pacientes bajo este régimen terapéutico es la llamada lipodistrofia asociada a terapia HAART, más conocido como HALS (*HIV-1 Associated Lipodystrophy Syndrome*). La prevalencia de la HALS entre los pacientes tratados oscila, dependiendo del estudio, cerca del 50% de tratados afectados, lo cual indica una alta importancia de este síndrome[312;313]. Últimamente con la introducción de los nuevos fármacos el porcentaje de afectados por este síndrome tiende a descender.

Las alteraciones en el tejido adiposo que se sufren bajo este síndrome son una lipodistrofia periférica del tejido adiposo subcutáneo (sobre todo en cara, extremidades y nalgas), una acumulación de grasa a nivel de tejido adiposo visceral y aparición en zonas aisladas de acumulaciones lipomatosas entre las que destaca por su prevalencia la zona dorso-cervical en las conocidas como gibas de búfalo o en inglés *Buffalo Humps* (BH). Además diferentes alteraciones metabólicas acompañan a esta redistribución de grasa. Estas alteraciones adiposas no suceden necesariamente de manera conjunta en el mismo paciente y la frecuencia de cada una de ellas es diferente. La más habitual, y que aparece en la mitad de pacientes tratados, es la lipodistrofia periférica que en la gran mayoría de casos va acompañada de una adiposidad central o acumulación de grasa a nivel visceral, siendo más reducida la aparición de lipomatosis[314]. Además de esta pérdida o acumulación de grasa en el tejido adiposo anatómicamente específica, los pacientes que sufren HALS presentan alteraciones o complicaciones metabólicas entre las que destacan la dislipidemia y la resistencia a insulina, así como un elevado riesgo de sufrir enfermedades cardiovasculares[314;315].

Figura-16. Alteraciones del tejido adiposo en pacientes infectados por HIV bajo terapia HAART que sufren HALS. De izquierda a derecha, lipoatrofia del tejido adiposo subcutáneo facial, lipohipertrofia del tejido adiposo visceral y lipomatosis en la zona dorso-cervical o *Buffalo-Hump* .

Entre los medicamentes que forman parte de las diferentes familias de fármacos que componen la terapia HAART, existen diferentes estudios cuyo objetivo es comprender en qué medida favorece cada uno de ellos la aparición de los diferentes efectos secundarios conocidos. Los primeros estudios que se realizaron describieron que los NRTIs favorecían la lipodistrofia y que los PIs estaban más relacionados con la adiposidad visceral y con la aparición de complicaciones metabólicas. Estudios más recientes proponen, teniendo en cuenta las complicadas combinaciones de fármacos que se dan en la terapia HAART, un síndrome lipodistrófico provocado por la sinergia de fármacos de las diferentes familias y que además van asociadas a alteraciones provocadas por la propia infección por el HIV[315].

Es interesante comentar que además de esta lipodistrofia asociada a la terapia HAART y otras lipodistrofias adquiridas por diversos motivos, existen lipodistrofias congénitas. Estas son poco frecuentes y presentan diferentes formas de expresión. La lipodistrofia generalizada congénita (o síndrome de Bernardinelli-Seip) afecta a pacientes que presentan una perdida completa del tejido adiposo. Puede ser de dos tipos 1 y 2, siendo la primera debida a una mutación en el gen de AGPAT2 (gen involucrado en la formación de triglicéridos y fosfolípidos) y la segunda en el de la Seipina cuya función está aún en debate[316]. También existen lipodistrofias congénitas parciales, en las cuales normalmente se pierde la grasa de las extremidades, como la lipodistrofia familiar parcial de Dunnigan, en la que se ven involucradas mutaciones en los genes de las laminas A y C[317]. Entre las lipodistrofias parciales también podemos encontrar la lipodistrofia asociada a mutaciones en PPARγ o la asociada a la displasia mandibuloacral. En muchos casos estas lipodistrofias congénitas conllevan también disfunciones metabólicas como hipertrigliceridemia, hiperinsulinemia y diabetes mellitus así como esteatosis hepática.

4. B. ALTERACIONES EN EL TEJIDO ADIPOSO DURANTE EL HALS

Se han realizado diferentes estudios para conocer las alteraciones que llevan al tejido adiposo subcutáneo a sufrir la característica lipoatrofia que se da en los pacientes bajo régimen HAART. De hecho, como se verá más adelante, algunos de los objetivos de esta tesis han sido determinar estas alteraciones y conocer su origen.

A nivel **morfológico**, diferentes biopsias de tejido adiposo subcutáneo de pacientes bajo terapia HAART presentaban en diferentes análisis microscópicos características histopatológicas comparadas con biopsias de individuos sanos. Entre estas características destacaban: adipocitos más pequeños que los de los individuos sanos, un mayor nivel de fibrosis y presencia de adipocitos en estado apoptótico[318;319], así como señales de inflamación como infiltración de macrófagos o lipogranulomas[319]. También han sido descritas alteraciones a nivel mitocondrial como un alto número de mitocondrias con estructuras anormales[320].

Uno de los puntos importantes del estudio de las afecciones provocadas por la terapia HAART en el HALS, han sido los estudios de las alteraciones a nivel de **expresión génica de genes relacionados con la adipogénesis** y genes dependientes de estos. En estos estudios las características observadas a nivel morfológico se han confirmado. Se ha descrito que diferentes fármacos son capaces de inhibir la adipogénesis como se muestra en el apartado de resultados**.** Los mecanismos por los que los diferentes fármacos son capaces de disminuir la adipogénesis en el tejido adiposo subcutáneo no están claros, pero un hecho bien descrito es que en pacientes que sufren HALS se da una expresión de genes *master* de la adipogénesis como son PPARγ y C/EBPα, anormalmente baja[319;321]. Además se han descrito alteraciones en los niveles de SREBP1 que en última instancia parecen conducir a un aumento y una acumulación de la forma inactiva de la proteína que es incapaz de translocarse al núcleo[322]. Una consecuencia de estas alteraciones en la expresión de estos genes es un descenso en la expresión de genes relacionados con la diferenciación adipogénica. De hecho se han descrito disminuciones en las expresiones por ejemplo de LPL o de GLUT4[321;322] y esto tiene como consecuencia para la célula adipocitaria un descenso en la captación de ácidos grasos y de glucosa. Esto conlleva una disminución en la acumulación de lípidos por parte del adipocito. Además y probablemente relacionado con el descenso de expresión de genes adipogénicos se da una reducción en la expresión de las adipoquinas adiponectina y leptina[321;322].

Además de este descenso en genes relacionados con la adipogénesis, una de las primeras hipótesis para explicar las causas del HALS fue la **toxicidad mitocondrial** que se ha descrito sobre todo para los NRTI[323;324]. Estos fármacos (en especial la estavudina y menor medida la zidovudina) son capaces de inhibir la polimerasa-γ mitocondrial (enzima encargado de la replicación del mtDNA) por el mismo mecanismo por el que inhiben la transcriptasa reversa vírica[325]. Este hecho provoca que los pacientes en

terapia HAART sufran depleción en el mtDNA (DNA mitocondrial) en diferentes tejidos siendo el tejido adiposo uno de los afectados[323;324]. Esta depleción implica un funcionamiento anormal de la cadena respiratoria que conlleva una disminución de ATP, así como un descenso en los niveles de UCP2, proteína desacopladora relacionada con la producción de especies reactivas de oxígeno (ROS)[321]. La importancia de la mitocondria en el tejido adiposo blanco había sido postergada tradicionalmente a un plano secundario probablemente debido a su escasa presencia en este tejido. Los resultados obtenidos en estudios relacionados con el HALS han contribuido a que se vuelva a valorar la importancia de la mitocondria en este tejido. De hecho hay estudios que describen la mitocondria como un complemento clave en la respuesta metabólica del adipocito a sensibilizadores a insulina como la rosiglitazona[326]. También se ha observado un descenso de la expresión de PGC1α, gen *master* regulador de la mitocondriogénesis, en el tejido adiposo de pacientes obesos[327] así como un descenso en la expresión de genes mitocondriales de cadena transportadora de electrones (OXPHOS) en obesos con diabetes tipo II[328].

Volviendo al HALS, se ha descrito que los niveles de lipodistrofia en los pacientes se correlacionan con los niveles de depleción de mtDNA que presentan y que si se da un cambio en la terapia o *switch* retirando el uso de la estavudina (altamente relacionada con la depleción del mtDNA) por otro fármaco, sea de la misma o de distinta familia, se aprecia una recuperación de esta depleción así como una moderada mejoría en la lipodistrofia que sufre el paciente[329]. Este hecho también ha sido observado en preadipocitos en cultivo tratados con fármacos de la HAART que provocan depleción, en los cuales se ha observado una incapacidad de los preadipocitos para diferenciarse[330]. Estos estudios indican que las alteraciones mitocondriales son capaces de provocar efectos importantes en el tejido adiposo que pueden ser importantes a la hora de comprender las alteraciones que sufre este tejido durante el HALS.

Una de las consecuencias conocidas de un metabolismo energético alterado por parte de la mitocondria, tal y como ocurre en el HALS o la obesidad, es la producción de especies reactivas de oxígeno (ROS, *Reactive Oxygen Species*) provocando un estrés oxidativo permanente en la célula, el adipocito en este caso[331]. Estos subproductos de la mitocondria son capaces de evitar la proliferación adipocitaria y su diferenciación[332]. Del mismo modo este estado de estrés oxidativo puede conducir a estados de inflamación y a la activación de la apoptosis.

Un hecho común en el tejido adiposo de pacientes sufriendo HALS es la aparición de una activación de la **apoptosis** en los adipocitos. Este hecho se ha relacionado, igual que la toxicidad mitocondrial, con el NRTI estavudina, ya que se ha descrito un aumento de apoptosis en el tejido adiposo de pacientes tomando este fármaco[329] del mismo modo que la retirada del mismo provoca un descenso de los niveles de

apoptosis así como a la ya mencionada mejora en la depleción en el mtDNA[329]. Esta apoptosis que sufre el tejido adiposo subcutáneo de estos pacientes se ha relacionado, lógicamente, con las vías apoptóticas mediadas por la mitocondria y de hecho, se ha descrito una alteración morfológica mitocondrial en adipocitos en apoptosis[333]. Los niveles de AMP provocados por las alteraciones mitocondriales comentadas más arriba, puede llevar a una activación de la AMP-quinasa (*AMP-Kinase*) que ha sido descrita como inductora de apoptosis en adipocitos[334]. Además la producción de ROS y el consecuente estrés oxidativo puede activar las vías de apoptosis[314]. Del mismo modo las adipoquinas inflamatorias también pueden jugar un papel importante en este proceso.

A nivel de tejido adiposo subcutáneo de pacientes con HALS se ha determinado que existe en este cuando se desarrolla una atrofia, un estado de **inflamación local**. En especial se han descrito altos niveles de expresión en el tejido adiposo subcutáneo de pacientes sufriendo HALS de TNFα y otras citoquinas inflamatorias como IL6 o IL18[319;335]. Este hecho viene acompañado por un aumento a nivel sistémico de de citoquinas inflamatorias como TNFα, interleuquinas 1,6,8 y 18 así como el interferón-α[336;337]. Además hay evidencias de infiltración de macrófagos en el tejido adiposos subcutáneo de estos pacientes en el que también se aprecia la presencia de lipogranulomas[318;319]. También se ha descrito un aumento de MCP1 en este tejido[338], hecho que provoca una mayor atracción de macrófagos que lleva a un aumento de señalización inflamatoria incluyendo un aumento en la propia MCP1. Este estado de inflamación local puede ser provocado por los propios fármacos de la terapia HAART como algunos NRTIs o PIs que han sido descritos como inductores de la expresión de TNFα e IL6, así como por la propia infección del virus HIV y/o sus proteínas virales capaces de inducir estas mismas citoquinas proinflamatorias[314]. Hay que recordar que la infección por el virus HIV activa las células endoteliales capaces de producir citoquinas como MCP1[339]. De esta manera parece ser una compleja suma de los efectos de la terapia y de la propia infección lo que está detrás del ambiente inflamatorio en este tejido.

Una alteración relacionada con este estado inflamatorio que se ha observado en el tejido adiposo subcutáneo atrófico de los pacientes con HALS, es un **aumento de lipólisis** y de niveles de ácidos grasos en circulación[340]. El aumento de TNFα y otras citoquinas como interleuquina-6 es capaz de aumentar la lipólisis en adipocitos[341;342], hecho que se relaciona con un aumento de FFAs (ácidos grasos libres no esterificados) liberados por el tejido adiposo de pacientes lipodistróficos en terapia HAART[343]. Este aumento de FFAs parece jugar un papel importante en la en la resistencia a insulina que sufren estos pacientes[343]. La activación de esta lipólisis parece ser una combinación de los efectos de la infección por el virus HIV-1 y alteraciones asociadas a terapia HAART. Se ha descrito que los fármacos de la familia de los PIs aumentan la lipólisis en adipocitos en cultivo[344], y también se ha observado que la retirada de

fármacos de esta familia en pacientes trae como consecuencia una reducción de la actividad lipolítica así como un descenso en los niveles de FFAs[345].

4. C. IMPLICACIÓN DEL VIRUS HIV-1 EN LAS ALTERACIONES ASOCIADAS A HALS

Diferentes estudios indican que las alteraciones observadas en el tejido adiposo subcutáneo de los pacientes de terapia HAART que sufren el síndrome lipodistrófico, así como los efectos metabólicos a nivel sistémico no pueden ser explicados por los fármacos de la terapia HAART por sí solos. De hecho existen estudios en el subgrupo de pacientes infectados por el virus HIV-1 que aún no reciben terapia anti-retroviral, conocidos como pacientes *Naive* que muestran que estos pacientes ya presentan alteraciones reminiscentes de lipodistrofia[314]. Por ejemplo se han observado en estos pacientes naive signos de depleción de mtDNA y sus consecuencias[314], reducción en la expresión de genes relacionados con la diferenciación como PPARγ o reducción de genes que expresan adipoquinas como adiponectina[321]. También se ha visto un aumento de la expresión de TNFα en el tejido adiposo de estos pacientes naive[321].

Todas estas alteraciones aparecen completamente desarrolladas en los pacientes que sufren el síndrome lipodistrófico asociado a la terapia HAART, pero parece que la infección por el virus es capaz de desencadenar los primeros signos de estas alteraciones que pueden ser amplificadas por la terapia HAART para acabar de definirse en un síndrome lipodistrófico.

La capacidad de los adipocitos para ser infectados por el virus HIV-1 no está clara, a pesar de que existen estudios que afirman que los adipocitos pueden ser susceptibles a esta infección[346]. Pero hay que tener en cuenta que el tejido adiposo está compuesto por diferentes tipos de células como los macrófagos, que juegan un papel importante en las alteraciones del tejido adiposo, y que sí pueden ser infectadas, hecho que puede tener importantes consecuencias en su entorno debido a las moléculas señalizadoras que liberan[347].

Además de la acción directa del virus, hay que tener en cuenta que la infección de un paciente por el HIV-1 hace que el virus libere proteínas víricas como Vpr o Tat. Estas proteínas pueden tener efectos en células que no sufren la infección por parte del virus[348;349]. Se ha descrito que Tat en diferentes tipos celulares, induce la inflamación como las vías pro-apoptóticas[349]. Del mismo modo se ha descrito que Vpr inhibe la sensibilidad a insulina, o altera el metabolismo lipídico sistémico y también se relaciona con la apoptosis vía activación de MPTP (*Mitochondrial Permeability Transition Pore*)[348].

4. D. ALTERACIONES EN LA SECRECIÓN DE ADIPOQUINAS EN EL HALS

Las alteraciones que sufre el tejido adiposo de los pacientes en HALS por la suma de la propia infección y el tratamiento HAART afectan a todos los ámbitos de función del tejido adiposo. Una de estas funciones, tal y como se ha explicado antes, es la secreción de adipoquinas que están relacionadas con la homeostasis metabólica de manera autocrina o paracrina. Esto implica que las alteraciones a este nivel pueden provocar disfunciones metabólicas, como las que se observan en el HALS.

Las adipoquinas sobre las que se han centrado la mayoría de estudios han sido leptina y adiponectina. La leptina se correlaciona con la masa de tejido adiposo del individuo en la población general. Este hecho se reproduce en los pacientes con HALS y cuanta más pérdida de tejido adiposo presentan por la lipodistrofia que les afecta, más bajos son sus niveles de leptina[350]. Los niveles de adiponectina, en cambio, se correlacionan inversamente con la cantidad de masa adiposa en individuos sanos. Pero en los pacientes lipodistróficos se aprecia un descenso en los niveles de adiponectina que se correlaciona inversamente con los triglicéridos en suero y la resistencia a insulina[319;350;351].

También se ha observado que los niveles de expresión de estos dos genes en el tejido adiposo aparecen disminuidos respecto a individuos control[322]. Probablemente en este hecho juegue un papel importante la disminución de expresión de PPARγ en el tejido adiposo de estos pacientes[321]. La aumentada expresión de citoquinas pro-inflamatorias es un conocido represor de la expresión de PPARγ, y este hecho sumado a la disfunción mitocondrial y el estrés oxidativo que sufren estos pacientes puede ser la causa de la disminución en la expresión de los genes como la secreción de estas adipoquinas[352].

4. E. LIPOHIPERTROFIA, LIPOATROFIA Y LIPOMATOSIS; DIFERENTES ALTERACIONES DEL TEJIDO ADIPOSO EN EL HALS DEPENDIENTES DE DISTRIBUCION ANATOMICA

Como se ha comentado anteriormente, el tejido adiposo de los pacientes infectados por el virus HIV-1 tratados con HAART que sufren el síndrome lipodistrófico asociado a este tratamiento, sufre diferentes alteraciones morfológicos como son: una lipomatosis y una lipoatrofia asociadas al tejido adiposo subcutáneo, así como una lipohipertrofia asociada al depósito adiposo omental o visceral. Durante este trabajo de tesis en los últimos años, uno de los objetivos ha sido determinar cómo y en qué medida afectan las alteraciones moleculares y metabólicas a este comportamiento diferente de los diferentes depósitos y las diferentes zonas anatómicas de un mismo depósito adiposo, como es el subcutáneo.

Tal y como se ha explicado con anterioridad, un sub-grupo de pacientes que sufren HALS presentan lipomatosis que habitualmente (del 6 al 13% de prevalencia en pacientes con HALS) se desarrolla en la zona dorso-cervical[314]. Estas lipomatosis o acumulaciones de grasa son conocidas como *Buffalo-Humps* (BH). Además también se han observado acumulaciones de este tipo en diversas localizaciones anatómicas como el pubis, el tronco o cuello, pero su prevalencia es más baja que las BH. Es importante mencionar que estas acumulaciones se dan a nivel de tejido adiposo subcutáneo, un depósito que por otra parte se caracteriza por desarrollar una lipoatrofia en los pacientes lipodistróficos, indicando que este depósito actúa de manera diversa anatómico-específicamente. Este hecho indica que la respuesta a las alteraciones provocadas por el tratamiento y la propia infección del virus HIV-1 dependiendo de la zona anatómica puede ser diferente.

Se ha observado que los adipocitos de las BH son pequeños y presentan un patrón de distribución vacuolar diverso, mostrando adipocitos univacuolares así como multivacuolares[353]. Otro hecho conocido es que estas zonas lipomatosas son capaces de expresar UCP1[354], hecho que acompañado de las alteraciones morfológicas sugiere que estos adipocitos sufren la adquisición de un fenotipo con algunas características *brown-like*.

Resulta interesante destacar que el auto-trasplante de tejido adiposo lipomatoso de la zona dorso-cervical a la zona lipoatrófica de la cara en los pacientes con HALS derive en algunos casos en el denominado síndrome hámster que consiste en un crecimiento de este tejido adiposo trasplantado en una zona característicamente lipoatrófica[355]. Este hecho indica que los adipocitos trasplantados desde un BH mantienen su alta capacidad proliferativa incluso en un ambiente lipoatrófico.

Por otra parte está la diferencia existente entre la lipohipertrofia que se da en el tejido adiposo visceral y la lipoatrofia del tejido adiposo subcutáneo. En este caso el comportamiento diferencial de ambos se puede atribuir a que se trata de dos depósitos con diferencias sustanciales en su biología y que ya en alteraciones como la obesidad se asocian de manera diversa a las alteraciones patológicas metabólicas asociadas. (ver apartado 3. B. VII.). En el HALS este comportamiento distinto de ambos depósitos se ha propuesto que viene asociado a diferentes fármacos del régimen HAART: Los PIs se asocian a la lipohipertrofia visceral mientras los NRTI son comúnmente asociados a la lipoatrofia subcutánea. Esto va en sintonía con el hecho de que la mayoría de alteraciones metabólicas durante el HALS se asocian a los PIs, del mismo modo que se asocian a la adiposidad visceral debido a su resistencia a insulina[194;356]. Se desconoce como los distintos aspectos de la etiopatología del HALS (la toxicidad mitocondrial, la infección por el HIV-1, la inflamación y un aumento de lipólisis) contribuyen a explicar la diferente respuesta de estos dos depósitos ante este síndrome.

4. F. LIPOTOXICIDAD COMO BASE DE LAS ALTERACIONES METABÓLICAS ASOCIADAS A HALS

La teoría de la lipotoxicidad intenta explicar cómo se provoca el desarrollo de alteraciones metabólicas en situaciones patológicas como la obesidad o las lipodistrofias. La lipotoxicidad es un modelo que explica como una alta disponibilidad de ácidos grasos o la incapacidad para metabolizarlos en diferentes órganos y tejidos, como el hígado, el musculo esquelético o el páncreas, provoca en ellos disfunciones y conduce a estas alteraciones metabólicas como la resistencia a insulina[357]. De esta manera la toxicidad provocada por estos ácidos grasos en las células-β puede llevar a una producción alterada de insulina, la acumulación de estos ácidos grasos en el musculo esquelético puede llevar a una resistencia a la insulina y en el hígado, un exceso de lípidos puede generar las múltiples disfunciones metabólicas asociadas al hígado graso. Todas estas alteraciones pueden provenir de disfunciones en el metabolismo lipídico o de una alta ingesta de grasa[358], pero principalmente serian debidas a una saturación de la capacidad del tejido adiposo para almacenar grasa.

La lipotoxicidad puede ser una de las bases para explicar las disfunciones metabólicas que sufren los pacientes con HALS ya que el hecho de la limitada capacidad de acumular lípidos del tejido adiposo subcutáneo lipoatrófico puede contribuir a la distribución ectópica de estos ácidos grasos. Apoyando esta teoría, se ha descrito comúnmente esteatosis hepática asociada a resistencia a insulina en los pacientes que sufren HALS, así como acumulaciones de grasa en el músculo esquelético que se asocian con un descenso de toma de glucosa por este tejido[358].

Como se ha comentado los NRTIs se han relacionado comúnmente con la lipodistrofia periférica y la toxicidad mitocondrial en tejido adiposo subcutáneo. Una de las consecuencias de la toxicidad mitocondrial es el descenso de la oxidación de los ácidos grasos en el tejido adiposo subcutáneo. Además hay diferentes estudios que relacionan el uso de esta familia de medicamentos con el aumento de lipólisis en el depósito subcutáneo, provocando la liberación de ácidos grasos no esterificados. Los PIs, por su parte, se han asociado a alteraciones metabólicas como la resistencia a la insulina tal y como se ha comentado anteriormente. También se ha visto que son capaces de impedir la adipogénesis y de promover la lipólisis en el tejido adiposo, y esto contribuye, lógicamente a la liberación de ácidos grasos por parte de este tejido contribuyendo a la acción lipotóxica. Además también hay que tener en cuenta que la propia infección por parte del virus HIV-1 y sus proteínas víricas asociadas, como se ha mencionado, son capaces de provocar cambios en la diferenciación adipogénica y en la lipólisis, contribuyendo también a esta lipotoxicidad.

Todos estos factores implicados en el HALS como son los diferentes fármacos y sus combinaciones y la propia infección por el HIV-1 generan alteraciones secundarias que son capaces de promover la lipotoxicidad. Tanto las proteínas víricas como los

fármacos están relacionados con la inhibición de la expresión de genes *master* de la adipogénesis como es PPARγ, tal y como se ha mencionado en apartados anteriores. Este hecho provoca disfunciones en los adipocitos y limita la expandibilidad del tejido adiposo. El ambiente pro-inflamatorio del tejido adiposo y la liberación de citoquinas pro-inflamatorias también contribuyen directamente a la represión de los genes *master* de la adipogénesis. Como consecuencia de la represión de estos genes que controlan la adipogénesis se da la represión, entre otros, de la expresión de LPL en el tejido adiposo subcutáneo reduciendo la capacidad de este para absorber ácidos grasos.

Todos estos acontecimientos están relacionados con una alta disponibilidad en órganos y tejidos como los citados hígado, páncreas o músculo esquelético, de ácidos grasos. Esta alta disponibilidad o una baja capacidad para oxidarlos pueden provocar alteraciones que son altamente comunes en los pacientes que sufren HALS. Por lo tanto la lipotoxicidad es un mecanismo capaz de explicar cómo las disfunciones asociadas a los fármacos de la HAART o la infección por el virus HIV-1 que afectan al tejido adiposo, pueden contribuir a las alteraciones metabólicas que se observan en los pacientes tratados que sufren el síndrome lipodistrófico[358].

5. FGF 19 Y FGF21 NUEVOS FACTORES INVOLUCRADOS EN EL CONTROL DE LA HOMEOSTASIS METABOLICA

5. A. INTRODUCCIÓN

Tal y como se ha insistido a lo largo de esta introducción, la homeostasis energética es un mecanismo crítico para la supervivencia. Por ello existen diferentes mecanismos hormonales capaces de controlar, regular y coordinar el uso energético a nivel celular así como a un nivel más alto como la comunicación entre los diferentes tejidos. Entre ellos son bien conocidos la insulina o el glucagón por ejemplo. Pero recientemente se ha identificado un nuevo subgrupo de FGF (*Fibroblast Growth Factors*) que parecen jugar un importante papel en la red regulación metabólica. Se trata de los componentes de la subfamilia filogenética FGF19 que incluye los FGF 19, 21 y 23. Esta subfamilia también es conocida como subfamilia de FGFs atípica ya que sus tres miembros presentan características que difieren de las del resto de las subfamilias de FGFs más clásicas[359].

Los *Fibroblast Growth Factors* provocan sus efectos uniéndose a los receptores de FGF (FGFR) en las superficies celulares. Los FGFRs son receptores tirosín quinasas codificado por 4 genes FGFR1, 2, 3 y 4, de los cuales en los tres primeros hay *splicing* alternativo que puede dar dos isoformas la *b* y la *c* que difieren en su dominio extracelular[360]. Los FGFs convencionales requieren una interacción con proteoglicanos heparán sulfato para así poder activar sus receptores FGFRs. La unión de los FGFs clásicos a los complejos FGFR/heparán sulfato provoca la dimerización de los receptores y una autofosforilación que conlleva una fosforilaciones de sustratos *downstream* que incluyen MAP quinasas como ERK1 y ERK2[360].

A diferencia de los FGFs convencionales los miembros de la subfamilia 19 de FGFs, no tienen afinidad por heparán sulfatos[361]. Como consecuencia de esto FGF19, 21 y 23 eluden las uniones a los proteoglicanos heparán sulfato extracelulares y así pueden ser difundidos por la circulación funcionando de manera endocrina. El hecho de carecer de afinidad por heparán sulfatos hace que para compensarlo los FGFs endocrinos necesiten miembros de la familia de proteínas Klotho para su unión a receptor FGFR[362]. Hay tres Klothos: α, β y *lactase-like*. Las 3 son proteínas de único paso transmembrana que interaccionan con FGFRs para la unión de los FGFs endocrinos. De manera selectiva α-Klotho o Klotho simplemente, funciona como correceptor para FGF23 y en cambio β-Klotho es correceptor para FGF19 y FGF21[363]. Los FGFR presentan una distribución extensa por todos los tejidos, en cambio los correceptores Klotho/βKlotho son más restrictivos en cuanto a su distribución; Klotho aparece en riñones, cerebro y órganos reproductivos mientras β-Klotho se expresa en tejido adiposo, hígado y páncreas[364]. Este hecho determina la especificidad tisular de los FGFs endocrinos.

En este estudio de tesis se tendrán en cuenta los FGFs FGF19 y FGF21 por su relación con las alteraciones metabólicas que se asocian a los pacientes infectados por el virus HIV-1 bajo tratamiento antirretroviral que sufren lipodistrofia asociada a este tratamiento.

5. B. FGF19 Y METABOLISMO

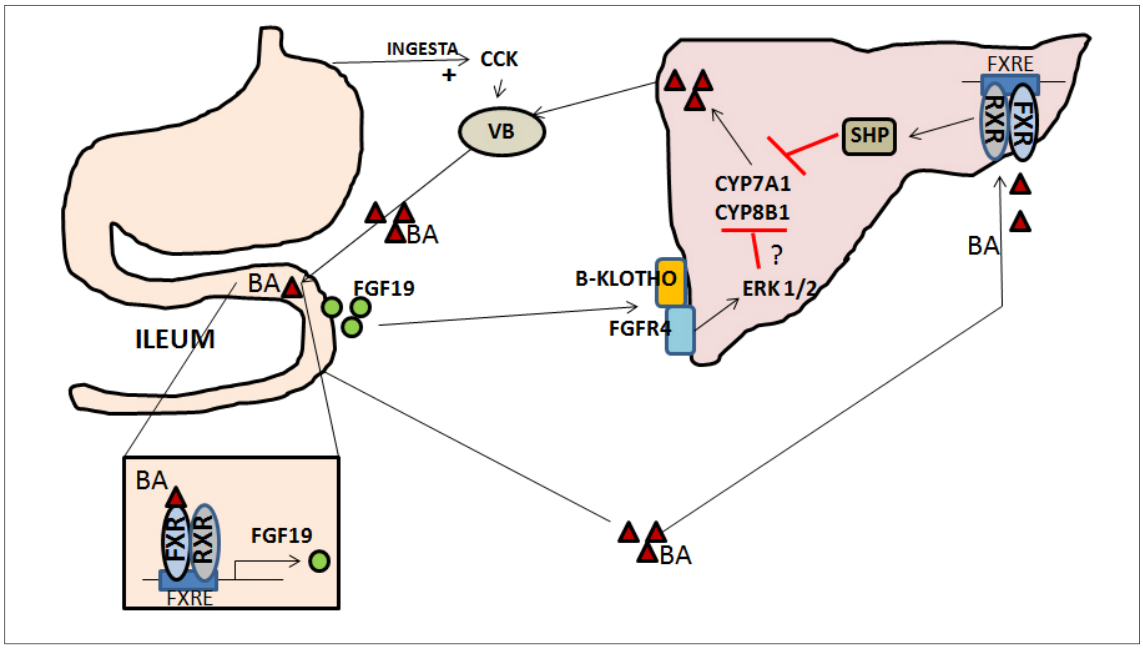
La expresión de FGF19 en humanos se detecta en el cerebro, cartílago, piel, retina, vesícula biliar y sobre todo en el intestino. A pesar de que los FGFs en general están altamente conservados entre ratones y humanos, compartiendo más del 90% de los aminoácidos, el caso de FGF19 es diferente y su ortólogo en ratón (FGF15) solo comparte un 50% de la identidad aminoacídica[365]. Aún así los genes de *FGF19* y *Fgf15* presentan un patrón de expresión tisular semejante y las proteínas para las que codifican provocan efectos similares en la expresión génica y en los parámetros metabólicos en ambas especies[366].

Una de las funciones principales de FGF19 es la regulación de la homeostasis de ácidos biliares. Los ácidos biliares son liberados post-pandrialmente por la vesícula biliar en el intestino delgado para solubilizar los lípidos de la dieta y así permitir su absorción[367]. Una vez atravesado el intestino un 95% de los ácidos biliares son reabsorbidos en el íleon y vuelven al hígado y la vesícula vía vena porta. Uno de los reguladores transcripcionales de los ácidos biliares es FXR (*Farnesoid X Receptor*) miembro de los factores de transcripción asociados a ligando de la familia de los receptores nucleares que es activado por algunos ácidos biliares[368]. Entre los genes que regula FXR en el hígado, está CYP7A1 (*Cholesterol 7α-hydroxylase*) un enzima que regula la síntesis de ácidos biliares en el hígado[369]. FXR puede reprimir la expresión de CYP7A1 y por los tanto inhibe la síntesis de ácidos biliares, de dos maneras diferentes. En una de ellas, la primera, la unión de ácidos biliares a FXR induce la expresión hepática de SHP (Small Heterodimer Partner) un receptor nuclear huérfano que se une al promotor de CYP7A1 y reprime su transcripción[370]. SHP no posee dominio de unión a DNA por lo tanto se une al promotor de CYP7A1 indirectamente por interacciones con otros receptores nucleares huérfanos como LRH-1 (*Liver Receptor Homolog 1*) y HNF4α (*Hepatocyte Nuclear Factor 4α*)[370;371]. Esta represión de CYP7A1 por la vía FXR-SHP se ha descrito en diferentes experimentos en los que ratones FXR-KO y SHP-KO presentan un incremento en la expresión de CYP7A1 y un correspondiente aumento en el pool de ácidos biliares[372;373].

La segunda vía por la que FXR inhibe CYP7A1, implica a FGF19 (FGF15). En esta vía los ácidos biliares reprimen su propia síntesis por *feed-back*. Se observó que FGF19 inhibía la expresión de CYP7A1 en hepatocitos en cultivo y en ratones[373]. También se describió que los ratones FGF15-KO no sufrían estar represión de CYP7A1[366]. Tras esto se descubrió que FGF19 se expresa en las células entéricas del íleon en respuesta a la unión de los ácidos biliares con FXR y no en el hígado como se había pensado

inicialmente debido a que los hepatocitos en cultivo si son capaces de expresarlo[374]. Este hecho se ha observado al verse los niveles séricos de FGF19 aumentados en respuesta a dosis orales de ácidos biliares y disminuidos en respuesta a tratamientos con secuestradores de ácidos biliares[359]. Tras esto, FGF19 (FGF15) reprime CYP7A1 en el hígado uniéndose al heterodímero FGFR4/β-Klotho como se ha demostrado en ratones FGFR4-KO y β-Klotho-KO en los que tal y como sucedía con los FGF15-KO sufren un aumento de expresión de CYP7A1 y síntesis biliar. Además la administración de FGF15/19 exógena en estos ratones no revierte la situación[366;375]. También se ha observado que la sobre-expresión de FGFR4 en animales *wild-type* o FGFR4-KO disminuye la expresión de CYP7A1 y los niveles de ácidos biliares. Otro hecho destacable es que la represión mediada por FGF15/19 de CYP7A1 se pierde en ratones SHP-KO demostrando que las vías FGF15/19 convergen[359]. Una posibilidad de esta convergencia, cuyo mecanismo aún no está claro, propone que FGF15/19 aumente la estabilidad de SHP previniendo su ubiquitinación[376]. De todas formas un aumento del nivel basal de expresión de SHP tiene solo efectos relativamente modestos en la expresión de CYP7A1 comparado con FGF19/15. En humanos se ha determinado que en enfermedades en las que el aumento de ácidos biliares o su descenso provocan alteraciones, los niveles de FGF19 están alterados[359].

Figura-17. Esquema en el que se muestra el mecanismo por el que FGF19 regula la síntesis de ácidos biliares y el mecanismo de feed-back negativo de los propios ácidos biliares entre el intestino y el hígado. VB es vesícula biliar, BA ácidos biliares y CCK coleocistoquinina.



Además FGF19/15 también está implicado en la regulación del llenado de la vesícula biliar de cuyo vaciado durante la ingesta se encarga la coleocistoquinina. Los ratones FGF15-KO tienen una vesícula siempre vacía, incluso en el ayuno. Los ratones FGFR4-KO y los β-Klotho-KO presentan vesículas pequeñas en relación a los controles. Una administración de FGF19 recombinante a los ratones FGF15-KO permite un rápido llenado de la vesícula biliar[375].

Existen diferentes estudios que han puesto de manifiesto la importante participación de FGF19 en el control de la **homeostasis energética**. Uno de los primeros estudios fue el realizado con ratones transgénicos para FGF19[377]. Estos ratones son delgados y están protegidos contra la obesidad inducida por la dieta, además presentan bajo contenido en grasa a pesar de una alta ingesta, hecho que se relaciona con un aumento de gasto energético asociado a un aumento del tamaño de BAT y de un aumento en la oxidación de ácidos grasos en el hígado. Además presentan bajos niveles de glucosa, insulina, colesterol y triglicéridos en suero[377]. Con la administración de FGF19 recombinante a ratones normales en dieta *High-fat* se reproducen estos efectos metabólicos[378]. Relacionado con esto, los ratones FGFR4-KO presentan características de síndrome metabólico como dislipidemia, hipercolesterolemia, aumento de adiposidad e intolerancia a la glucosa[379]. Paradójicamente, teniendo en cuenta que FGF19/15 aumenta la oxidación de ácidos grasos y disminuye lipogénesis, los ratones obesos FGFR4-KO presentan una disminución de triglicéridos y colesterol a nivel hepático[379]. Una reconstitución a nivel hepático de FGFR4 en los ratones FGFR4-KO logra una disminución de los niveles de lípidos y colesterol en plasma pero no resuelve la intolerancia a glucosa ni la sensibilidad a insulina[379]. Además la administración de FGF19 a los ratones FGFR4-KO mejora la homeostasis de glucosa[380]. Este hecho indica que FGF19 requiere FGFR4-KO para su mejora de la dislipidemia e hipercolesterolemia, pero en cambio este receptor no parece importante para sus efectos sobre la homeostasis de glucosa y la resistencia a insulina. FGF19 presenta una mayor afinidad por FGFR4 en hígado, pero parece que también es capaz de unirse y activar FGFR1c con una alta afinidad, este receptor es altamente expresado en tejido adiposo blanco (WAT) pero no en hígado[364;381]. De acuerdo con este hecho un estudio llevado a cabo por Ge *et al*. (2012) logró una variante de FGF19, el FGF19-7 que ha perdido su capacidad de unirse a FGFR4 pero mantiene la capacidad de unirse y activar FGFR1c y que mantiene todas las características regulatorias a nivel de homeostasis de glucosa[382].

La relación de FGF19 con los adipocitos se observó en un estudio en el que se expusieron adipocitos directamente a FGF19 en cultivo. Se observó un aumento en la señalización FGF así como un aumento en la captación de glucosa por parte de los adipocitos de manera dependiente de β-Klotho induciéndose ERK1/2[364]. Este hecho indica que además de su conocido efectos sobre el receptor FGFR4 en hígado, FGF19 una vez en el torrente sanguíneo puede actuar uniéndose a diferentes FGFR en

diferentes tejidos[381]. Confirmando este hecho, inyecciones de FGF19 intracerebroventriculares provocan un aumento del ritmo metabólico indicando que FGF19 también puede actuar en el SNC[359].

Otro estudio que sugiere el papel de FGF19 en la homeostasis de la glucosa es el realizado con ratones FGF15-KO. Estos ratones son intolerantes a la glucosa y almacenan 50% menos glucógeno en hígado que los *wild-type*. Tras la administración de FGF19 se estimuló la síntesis de glucógeno hepática. En animales sin insulina la administración de FGF19 logró el mismo resultado indicando la independencia de la insulina de esta inducción de la síntesis de glucógeno hepática. También se observo en este estudio un aumento de síntesis proteica en el hígado de estos animales[383].

También ha sido observado que FGF19 reprime la gluconeogénesis. Ratones sobre-expresando FGF19 presentaban inhibición de la gluconeogénesis hepática sin verse afectada la sensibilidad a insulina. Se ha propuesto que FGF19/15 inhibe la gluconeogénesis inhibiendo la actividad del factor de transcripción CREB que entre otros genes gluconeogénicos, regula PGC1α[359].

Parece interesante que la similitud entre los efectos de la insulina y el FGF19, ya que ambos aumentan la síntesis de glucógeno y proteínas en hígado e inhiben la gluconeogénesis. Pero a pesar de esto FGF19/15 no estimula la lipogénesis como lo hace la insulina. Además actúan uniéndose a diferentes receptores. Y otro hecho diferencial entre ambos factores es que la insulina sufre un aumento inmediato tras la ingesta mientras que FGF19 presenta un pico de los niveles en suero dos horas tras la ingesta de manera que parece que FGF19 actúa tras la insulina en el paso del estado de ingesta al de ayuno. De acuerdo con este hecho los niveles en suero en humanos correlacionan negativamente con la glucosa en ayuno y el síndrome metabólico[359].

5. C. FGF21 Y METABOLISMO

El estudio de FGF21 en relación con sus acciones metabólicas comenzó en 2005 con un estudio que demostraba su capacidad sensibilizadora frente a insulina en ratones[290]. En este estudio y en otros posteriores con ratones genéticamente obesos o de obesidad inducida se ha observado que el tratamiento con FGF21 disminuye los triglicéridos hepáticos, también los triglicéridos en plasma, así como los niveles de glucosa. También disminuye el peso aumentando el gasto energético y disminuye la cantidad de grasa sin que se modifique la dieta[290;384;385]. Estudios parecidos en monos Rhesus diabéticos se observan efectos similares como son un descenso en plasma de insulina, glucosa, triglicéridos y colesterol LDL, mientras aumenta el HDL y una pérdida de peso[386]. Estos estudios determinan que FGF21 tiene importantes efectos sobre diferentes parámetros metabólicos entre los que se incluyen el metabolismo de

lípidos, de glucosa y profundos efectos sobre la sensibilidad a insulina, en ratones obesos y primates.

En ratones insulino-resistentes como son los ob/ob y los ratones con obesidad inducida por la dieta, el tratamiento con FGF21 disminuye los niveles de glucosa en sangre aumentando la tolerancia a glucosa y la sensibilidad a insulina[387], este efecto se da en los ratones obesos inducidos por la dieta sin disminución en la glucosa producida por el hígado. En cambio, estos ratones con obesidad inducida por la dieta, al ser tratados durante 3-6 semanas con FGF21 son capaces de revertir la esteatosis hepática, disminuyen la producción hepática de glucosa, aumenta la toma de glucosa en corazón, tejido adiposo y músculo esquelético de forma dependiente de insulina[384]. En cambio en los genéticamente obesos ob/ob, tratados con FGF21 durante 8 días se observa un aumento a sensibilidad hepática a insulina y un aumento de glucógeno en el hígado[388]. De todas formas no se apreció en estos ratones genéticamente obesos un aumento de absorción de glucosa insulino dependiente a nivel de tejidos periféricos. A pesar de que el motivo de esta diferencia no está claro, de ambos estudios se concluye que la mejora en la sensibilidad a insulina por el tratamiento con FGF21, en parte, es debido a efectos en el metabolismo hepático.

Para entender sobre qué tejidos actúa FGF21 es importante conocer los receptores sobre los que actúa. En este caso FGF21 actúa principalmente sobre FGFR1c, FGFR2c y FGFR3c, siempre acompañados por β-Klotho[363;389]. Mientras los tres FGFRs son ubicuos, la expresión de β-Klotho difiere entre tejidos y es elevada en algunos tejidos metabólicamente activos como son el hígado, el tejido adiposo blanco y el marrón[390]. Parece que FGF21 estimula la fosforilación de ERK1/2 y modula la expresión génica en estos tejidos[364]. Se conoce poco sobre esta regulación génica, pero se sabe que por ejemplo FGF21 disminuye los niveles del factor transcripcional lipogénico SREBP1 en hígado[384;391] y aumenta la expresión del coactivador PGC1α en hígado y WAT[391;392].

Otros tejidos en los que se expresa β-Klotho son el páncreas y el hipotálamo. En los islotes de páncreas de rata el tratamiento con FGF21 estimula ERK1/2 y Akt aumentando la expresión de insulina[393]. También en ratones db/db el tratamiento con FGF21 aumenta el número de islotes y los niveles de insulina. Esto parece indicar que el FGF21 preserva el funcionamiento de las células-β. En cuanto al hipotálamo son necesarios más estudios para determinar sus funciones, pero se puede pensar que los efectos de FGF21 pueden ser consecuencia de su acción a nivel central y periférico.

Estos estudios demuestran las acciones farmacológicas de FGF21 que básicamente tienden a sensibilizar ante la insulina a ratones con alteraciones o disfunciones metabólicas como son los ratones obesos o diabéticos. En cambio también se han realizado estudios para comprender los efectos fisiológicos de FGF21. Estos efectos se dan a concentraciones más bajas y en animales sin alteraciones metabólicas. Uno de los primeros estados fisiológicos en el que se describió una inducción de FGF21 en el

hígado de ratones, fue un **ayuno** prolongado durante al menos 12 horas[394]. Esta inducción, para ser óptima, requiere la presencia de receptores de glucagón[388], hecho que adquiere sentido al observarse que el FGF21 actúa tras el glucagón en la cascada de hormonas que controlan la respuesta al ayuno. De hecho también se ha observado que FGF21 puede disminuir la concentración de glucagón[290].

Esta inducción de FGF21 en hígado requiere la activación de PPARα, activado por los ácidos grasos o por fibratos (agonistas), y se une al promotor del gen de FGF21 para inducir su transcripción[395]. Existían estudios anteriores en los que se había observado que los ratones PPARα-KO no son capaces de catabolizar los ácidos grasos en el hígado y se vuelven esteatóticos, hipocetonémicos, hipoglicémicos e hipotérmicos durante el ayuno de al menos 24 horas. La hipocetonemia y la esteatosis se revierten con la administración de FGF21 externo[396]. En situaciones que mimetizan el ayuno, como ratones con dietas cetogénicas o, como se vio en nuestro laboratorio, ratones neonatos lactantes, también se observa una inducción de FGF21 en hígado[289;394].

Así, como se ha comentado, en ratones transgénicos para FGF21 se da una inducción hepática de PGC1α por el FGF21 estimulándose la transcripción de genes involucrados en la gluconeogénesis, oxidación de ácidos grasos y cetogenesis[359], procesos todos ellos que se observan durante el ayuno. Ratones knockdown para FGF21 en hígado bajo dietas cetogénicas no son capaces de oxidar ácidos grasos en el hígado sufriendo esteatosis hepática[394]. Se puede apreciar que las acciones de FGF21 se parecen a las del glucagón, pero el FGF21 no incrementa la glucogenolisis. Este hecho va de acuerdo con que el FGF21 es inducido tras 12-24 horas de ayuno, habiéndose acabado ya las reservas de glucógeno. Estos estudios indican que FGF21 juega un papel importante en la regulación de la producción de glucosa y el catabolismo de ácidos grasos durante el ayuno.

Como se ha comentado anteriormente, los ratones transgénicos FGF21 presentan alteraciones en el BAT que sugieren un papel importante de FGF21 en la **termogénesis adaptativa**[290]. De hecho, en nuestro grupo se ha descrito cómo FGF21 es inducido por el frio en el BAT de ratones[291]. De todas formas aún existe cierta controversia sobre si este FGF21 expresado por el BAT actúa a nivel local u hormonal. Lo que sí está claro es que los ratones obesos a los que se administra FGF21 pierden peso, tal y como se ha mencionado anteriormente. Esta pérdida de peso se relaciona con una activación de BAT en el que se induce la expresión de UCP1 o DIO2[289;385], genes termogénicos, en respuesta a FGF21 y también con un *browning* del WAT, ya que se induce UCP1 en este tejido[290]. Es curioso el hecho de que FGF21 dependiendo del contexto fisiológico (frio o ayuno) tome parte tanto en la bajada de temperatura durante el torpor como en el aumento de esta durante la termogénesis adaptativa. Este hecho parece asociarse a los tejidos expuestos a FGF21 en cada situación fisiológica, y también se observa un comportamiento similar en las catecolaminas.

Sorprendentemente FGF21 también aparece inducido en hígado durante la **ingesta** en dietas *high-carboydrates*[397] y en WAT durante regímenes *fasting-refeeding*[398]. Se ha descrito que ChREBP (Carbohydrate Response Element Binding Protein) y PPARγ, median esta respuesta en hígado y tejido adiposo blanco respectivamente[359]. En cuanto a PPARγ, recientemente se ha descrito que el FGF21 es necesario para la sensibilización a insulina total por la Rosiglitazona (TZD agonista de PPARγ), proponiéndose que FGF21 estimula PPARγ como mínimo previniendo su inactivación por sumoylización post-transcripcional[398]. En este sentido, es interesante comentar que los ratones FGF21-KO obesos inducidos por la dieta son resistentes a los efectos de sensibilización a insulina provocados por los TZDs así como a sus efectos secundarios.

Se ha propuesto que el FGF21 que se induce en el WAT durante la ingesta, a diferencia del secretado por el hígado durante el ayuno, no afecta a los niveles circulantes de FGF21[398]. Este hecho ha generado controversia sobre si el FGF21 generado por el tejido adiposo blanco durante la ingesta funciona de una manera más autocrina o paracrina como los FGFs clásicos. También existe controversia sobre la capacidad de FGF21 para provocar o evitar la lipólisis. Existen estudios que apuntan en ambas direcciones, indicando que los efectos de FGF21 en el WAT son complejos y dependen de contextos fisiológicos o patológicos[359].

El panorama varía ligeramente de ratones a humanos. Se ha descrito que tal y como sucede en ratones, en humanos los fibratos y otros agonistas PPARα inducen la expresión de FGF21[399]. También se ha apreciado un aumento de los niveles circulantes de FGF21 en pacientes durante 7 días de ayuno[399] y en obesos alimentados por una dieta de muy bajas calorías durante 3 semanas[400]. A pesar de estas similitudes el aumento de FGF21 es modesto en humanos en comparación con ratones. Además otros estudios indican que los niveles en suero de FGF21 no varían en dietas cetogénicas o ayunos no prolongados[399], indicando diferencias en la regulación y la función de FGF21 en humanos y roedores.

Del mismo modo resulta interesante la controversia existente sobre la capacidad del tejido adiposo blanco humano de expresar FGF21 ya que algunos grupos son capaces de detectarlo, a bajos niveles, mientras otros no[359]. Otro hecho interesante es el aumento de la cantidad de FGF21 en suero de pacientes con sobrepeso, diabéticos, intolerantes a glucosa o con esteatosis hepática[359]. Parece que este FGF21 proviene del hígado de estos pacientes, quizá por una inducción debida a elevados lípidos carbohidratos hepáticos. Este hecho se reproduce en ratones genéticamente obesos o con obesidad provocada por la dieta[401;402]. Es interesante que estos ratones aún responden a dosis farmacológicas de FGF21 mejorando su sensibilidad a insulina. Una explicación que se ha propuesto es que la obesidad y la diabetes puedan ser estados de resistencia a FGF21[402], a pesar de que existe controversia al respecto.

OBJETIVOS

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El objetivo general de esta tesis doctoral ha sido definir los diferentes mecanismos moleculares que provocan las alteraciones a nivel de tejido adiposo en los pacientes infectados por el virus HIV bajo una terapia HAART y que desarrollan el síndrome lipodistrófico asociado. Así mismo, durante el transcurso de esta tesis se ha identificado la importante función de unos nuevos factores relacionados con la homeostasis metabólica como son FGF19 y FGF21. De esta manera parte del trabajo reciente de esta tesis doctoral se ha orientado a definir la implicación de estos factores en la lipodistrofia asociada a HAART en pacientes infectados.

En particular, se abordan los siguientes objetivos específicos:

- Determinar en qué medida provocan algunos fármacos pertenecientes a terapia HAART utilizados en la actualidad (Efavirenz, Nevirapina, Lopinavir y Ritonavir) las alteraciones moleculares proclives a la lipodistrofia en adipocitos en cultivo.

- Estudiar las alteraciones de expresión génica diferenciales a nivel regional anatómico en áreas de tejido adiposo con distinto comportamiento en pacientes sufriendo HALS, consistiendo en:

    - Estudiar las bases moleculares que provocan un comportamiento diferencial en lipomas y tejido adiposo lipoatrófico (perteneciendo ambos al tejido adiposo subcutáneo) en estos pacientes lipodistróficos.

    - Estudiar, del mismo modo, el comportamiento diferencial del tejido adiposo subcutáneo lipoatrófico y el tejido adiposo visceral lipohipertrófico en pacientes con el HALS.

- Describir como se asocian los dos factores relacionados con la homeostasis energética FGF19 y FGF21, recientemente descubiertos, con las alteraciones metabólicas asociadas al síndrome lipodistrófico en pacientes bajo terapia HAART.

RESUMEN GLOBAL Y DISCUSION GENERAL

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Para estudiar y comprender los mecanismos moleculares relacionados con la lipodistrofia asociada a la terapia HAART, durante este estudio de tesis doctoral se han seguido diferentes estrategias que han quedado reflejadas en los artículos que conforman esta memoria. Teniendo en cuenta la experiencia de nuestro laboratorio en cuanto al tejido adiposo, estudios realizados anteriormente en nuestro grupo habían aportado datos respecto a diversos aspectos de la lipodistrofia asociada al tratamiento con antirretrovirales de alta actividad en pacientes infectados por el virus HIV-1, que han sido una robusta base para la actual tesis doctoral. Sumando las evidencias obtenidas en dichos estudios a los resultados descritos por otros grupos se habían asentado algunas bases de las diferentes alteraciones que sufre el tejido adiposo afectado por dicha lipodistrofia. Pero aún se debían y se deben caracterizar diversos mecanismos moleculares implicados en los efectos secundarios que esta terapia HAART provoca sobre el tejido adiposo de los pacientes para lograr, tanto una mejora de la terapia actual como una mejor comprensión de las disfunciones asociadas al tejido adiposo y sus consecuencias metabólicas, que juegan un papel tan importante en este síndrome así como en otros tan importantes y extendidos hoy en día como la obesidad.

Una de las importantes aportaciones realizadas anteriormente por nuestro grupo al respecto, entre otras, ha sido describir como las alteraciones que se observan en el tejido adiposo de los pacientes tratados que sufren lipodistrofia ya se observan en menor medida en pacientes *naive*, es decir, pacientes que han sido infectados pero que aún no han sido tratados con terapia HAART, así como en pacientes ya tratados que aún no han desarrollado la lipodistrofia. Este hecho indica que la propia infección vírica es capaz por sí misma de desencadenar algunas de las alteraciones típicas de este síndrome siendo uno de los primeros pasos hacia una futura lipodistrofia, a pesar de que esta se desarrolla más precipitadamente una vez entran en juego los fármacos antirretrovirales utilizados hoy en día y a los cuales se ha asociado tradicionalmente esta alteración[321].

La aportación individual de cada uno de estos fármacos antirretrovirales al desarrollo del síndrome lipodistrófico HALS es difícil de determinar debido entre otras cosas a su administración de forma combinada y a los diversos cambios que sufren los pacientes en el patrón de administración debido tanto a la resistencia del virus como a los efectos tóxicos provocados por estos medicamentos. De hecho, la administración combinada de los fármacos en el actual formato de la terapia HAART puede provocar que estos tengan un efecto sumatorio entre ellos a la hora de desencadenar los mecanismos que provocan el síndrome HALS. Uno de los objetivos de esta tesis doctoral ha sido determinar en qué medida contribuyen a las alteraciones en el tejido adiposo humano de forma individual algunos de los fármacos más utilizados hoy en

día. Teniendo en cuenta que resulta complicado determinarlo en los pacientes, para ello se han realizado estudios *in vitro* con preadipocitos humanos que han sido tratados individualmente con efavirenz (EFV), nevirapina (NEV) (dos NNRTIs utilizados), lopinavir y ritonavir (PIs que se administran conjuntamente en una proporción 4:1 conocida como kaletra (K)). La comparación entre los efectos que provocan en los preadipocitos en cultivo aporta una interesante información sobre cuál de estos fármacos es más proclive a desarrollar las disfunciones o alteraciones que desencadenan la lipodistrofia en los pacientes. Los estudios se organizaron de manera que se compararon directamente los efectos sobre los preadipocitos de los dos NNRTI, es decir el efavirenz con la nevirapina y por otra parte los efectos del propio efavirenz comparados con el lopinavir/ritonavir.

Uno de los puntos interesantes en el estudio de los fármacos directamente sobre preadipocitos es observar en qué medida son capaces de afectar, a concentraciones que no provoquen citotoxicidad, a la correcta diferenciación de los preadipocitos, ya que se ha descrito *in vivo* que uno de los acontecimientos principales que se observa en el tejido adiposo lipoatrófico es una inhibición de genes adipogénicos. El EFV no es uno de los fármacos tradicionalmente asociados a la lipodistrofia, pero a pesar de esto hay estudios recientes que indican que su presencia en los "cocktail" de la terapia HAART se correlaciona positivamente con la aparición de lipodistrofia en pacientes[83;403]. Los resultados obtenidos durante este estudio doctoral van de acuerdo con este hecho. Las observaciones a nivel de diferenciación de los preadipocitos indican que el EFV la reprime con mayor potencia que kaletra, que ya había sido descrito como inhibidor de la adipogénesis, y que la nevirapina que por el contrario parece no afectar a la diferenciación. Este hecho ya queda patente observando la morfología de los preadipocitos en cultivo: a partir de una concentración de 2µM de efavirenz quedan prácticamente indiferenciados. Para lograr esta misma represión es necesario llegar a casi 10µM de kaletra y en contraste, la nevirapina parece no afectar negativamente a la diferenciación, e incluso a 20µM de concentración de nevirapina se observa un aumento en esta diferenciación. De hecho había sido descrito cómo la nevirapina es capaz de promover la diferenciación impidiendo el crecimiento celular en diferentes tipos de células transformadas[404]. Esto ha llevado a proponer la nevirapina como tratamiento para algunos tipos de cáncer. Aún se desconocen los mecanismos moleculares por los que la nevirapina promueve la diferenciación contra la proliferación, pero la inhibición de la actividad transcriptasa reversa endógena ha sido una de las proposiciones que se han hecho ya que dicha actividad se asocia habitualmente a células en estado proliferativo y desaparece en el estado de diferenciación[405]. En el estudio de comparación entre el efavirenz y la nevirapina, hemos descrito por primera vez como la actividad endógena transcriptasa reversa se ve inhibida durante la diferenciación adipocitaria humana y que la nevirapina es capaz de inhibir precisamente esta actividad. Por otro lado, si bien se había descrito que en tipos celulares transformados el efavirenz también era capaz de

inhibir la actividad RT endógena, en preadipocitos humanos no se aprecia esta inhibición. Este hecho va de acuerdo con los efectos anti-diferenciación que observamos en el tratamiento con efavirenz y podría explicar, sumado a la represión de los principales genes reguladores adipogénicos los efectos opuestos que observamos con ambos tratamientos.

Además estos efectos se han visto confirmados a nivel de expresión génica. En este aspecto resulta interesante el hecho de que dos de los principales genes reguladores de la adipogénesis como son PPARγ y C/EBPα reproducen el patrón observado a nivel de diferenciación morfológica observado para los tres tratamientos, siendo el EFV el que los inhibe con mayor potencia a menores concentraciones. Ambos genes son conocidos por coordinar la adquisición tanto de una morfología adipocitaria como de un metabolismo característico de adipocitos. Por tanto la inhibición de estos dos genes es una de las bases por las que el efavirenz y el kaletra inhiben la adipogénesis. Además en el estudio comparativo entre el EFV y la NEV observamos que el SREBP-1, otro gen "master" para la adipogénesis también sufre una represión provocada por EFV en su expresión respecto a los cultivos control y los preadipocitos tratados con NVP. El mecanismo que provoca esta inhibición es desconocido pero queda patente observando los resultados que como consecuencia los genes característicos adipocitarios cuya expresión depende o es diana de los mencionados genes reguladores de la adipogénesis como son la lipoproteína lipasa (LPL), adiponectina o leptina también se ven reprimidos por el EFV y en menor medida por el K. En el estudio comparativo entre el efavirenz y el kaletra se confirmaron los resultados de expresión génica en adipocitos ya diferenciados indicando que el mecanismo por el cual el kaletra y en mayor medida el efavirenz son capaces de inhibir la expresión de estos genes es independiente del estado de diferenciación de los adipocitos.

Otro de los puntos que se estudiaron en estos cultivos fue la medida en la que los fármacos afectaban a la funcionalidad mitocondrial. Teniendo en cuenta que la capacidad de inhibir la adipogénesis y de provocar la lipodistrofia de algunos NRTI se ha asociado a la toxicidad mitocondrial que provocan, parece interesante determinar hasta qué punto son capaces el efavirenz y el kaletra de generar depleción del mtDNA y compararlo con la NVP, ya que si los dos primeros lo hicieran del mismo modo que algunos NRTIs como el AZT o la didanosina podría indicarnos el mecanismo aún desconocido por el cual provocarían esta represión de la adipogénesis. Los resultados a este respecto muestran que ninguno de los fármacos estudiados parece afectar los niveles de mtDNA ni la expresión de los genes codificados por este. Además, los niveles de lactato secretados por las células adipocitarias, indicador del funcionamiento oxidativo mitocondrial, no sufren cambio alguno respecto a los controles en ninguno de los casos, indicando un correcto metabolismo oxidativo mitocondrial. Este hecho indica que los efectos nocivos del kaletra y sobre todo, del efavirenz, o los efectos

levemente positivos de la nevirapina sobre los preadipocitos en cultivo ocurren en ausencia de toxicidad mitocondrial a diferencia de lo descrito para los NRTIs.

El estatus adquirido durante los últimos años por el tejido adiposo como un órgano endocrino acompañado de la reducida expresión que habíamos observado en los preadipocitos tratados con efavirenz y kaletra de las adipoquinas leptina y adiponectina, invitaba a estudiar como afectaban estos medicamentos a la secreción al medio de adipoquinas por parte de los preadipocitos. Confirmando los resultados obtenidos a nivel de expresión génica, la adiponectina y la leptina sufren un descenso en la secreción por parte de los preadipocitos tras ser estos tratados con efavirenz o kaletra y, en cambio, con el tratamiento con nevirapina esta secreción se ve levemente aumentada en el caso de la adiponectina y no afectada para la leptina. Este hecho resulta muy interesante teniendo en cuenta que se han descrito niveles bajos de adiponectina en los pacientes infectados por el HIV-1 en tratamiento que sufren lipodistrofia[351]. Siendo la adiponectina un agente sensibilizador a insulina[406] el hecho de que el efavirenz y en menor medida el kaletra sean capaces de reprimir tanto su expresión como su consecuente secreción es un nexo importante entre estos fármacos y los potentes efectos diabetogénicos de la terapia HAART. Por otra parte existen estudios que relacionan los "switch" o cambios, en los "cocktail" que consisten en abandonar un NRTI o un PI y cambiarlo por nevirapina, en los que se observa una mejoría en los niveles de adiponectina circulantes y en la insulinemia[407], mientras otros estudios no perciben efectos[408]. También son conocidas e importantes las propiedades anti-inflamatorias de la adiponectina[406], de esta manera un descenso de la secreción de esta contribuiría a generar un ambiente más pro-inflamatorio y un aumento ayudaría a reducir el estado inflamatorio.

Ampliando el estudio de secreción se estudiaron los niveles de citoquinas pro-inflamatorias liberadas al medio por los preadipocitos provocados por los diferentes tratamientos. La experiencia de nuestro laboratorio y estudios descritos por otros grupos confirman que los pacientes sufren un estado de inflamación local en el tejido adiposo, así como un aumento de marcadores de inflamación sistémicos[319;335]. Este hecho es importante ya que esta inflamación local en el tejido adiposo parece jugar un importante papel en las alteraciones metabólicas de los pacientes[314]. Las citoquinas proinflamatorias liberadas por los adipocitos juegan un papel muy importante en este contexto. Hay que tener en cuenta que en nuestro grupo se ha observado que los pacientes infectados que aún no han llegado al umbral de carga vírica que determina el inicio del tratamiento HAART, o pacientes "naive" ya presentan un patrón de inflamación local en el tejido adiposo subcutáneo sin que éste haya sido provocado por los medicamentos[321]. Si bien es cierto que en general este estado se ve agravado al acumular meses de tratamiento HAART, resulta interesante describir el comportamiento de las células adipocitarias en ausencia de la infección vírica, en cultivo ante estos tres tratamientos que son ampliamente utilizados hoy en día. De

nuevo, el EFV y K, a bajas concentraciones en las que ya reprimían la adipogénesis, provocan importantes alteraciones en la secreción de citoquinas proinflamatorias. El efecto es siempre más potente con el efavirenz que induce un aumento de secreción de importantes citoquinas pro-inflamatorias como MCP-1, IL8, IL6, PAI-1 o HGF. En cambio el lopinavor/ritonavir o kaletra muestra una inducción de la secreción de estas citoquinas más leve viéndose aumentos significativos de liberación al medio solo para IL6 y HGF. Por el contrario la nevirapina, a una concentración más alta y a la que observábamos cierto efecto positivo en la adipogénesis, también presenta un efecto levemente anti-inflamatorio y no se observa una variación en la secreción de IL8 y MCP-1 pero se reduce la liberación de IL6 y HGF al medio comparado con los controles. El hecho de que el aumento de IL6 haya sido relacionado con la resistencia a insulina[409] nos vuelve a dirigir hacia un posible efecto beneficioso insulinémico de la nevirapina a esta concentración (20µM). Los niveles de HGF se ven aumentados en el tejido adiposo que sufre inflamación local de pacientes obesos[410], y este hecho hace que se utilice como marcador de inflamación local del tejido adiposo, por lo tanto la reducción de su secreción que se ve en el caso del tratamiento con la nevirapina, así como el aumento en el caso de EFV y K, van en consonancia con los demás resultados indicando que el efavirenz es el fármaco que con más potencia provoca un ambiente pro-inflamatorio en células adipocitarias en cultivo seguido por el kaletra y en contraste con ambos, la nevirapina parece mostrar cierto efecto anti-inflamatorio que podría ir asociado a beneficios en la homeostasis insulinémica.

Viendo los resultados de manera global, podemos decir que el efavirenz, el cual se tenía tradicionalmente como uno de los fármacos no nocivos para el tejido adiposo de la terapia HAART, es un potente inhibidor de la adipogénesis, que además reprime la secreción de adipoquinas antidiabéticas como la adiponectina y aumenta la secreción de citoquinas pro-inflamatorias. El kaletra presenta unos efectos negativos similares pero con un patrón más leve que el efavirenz. Por otra parte la nevirapina no tiene efectos negativos sobre los marcadores estudiados, si bien a una concentración determinada (20µM) parece provocar efectos levemente beneficiosos sobre la adipogénesis y sobre la liberación de adipoquinas antidiabéticas como la adiponectina. Por otro lado también reduce la liberación de citoquinas pro-inflamatorias hecho que puede reducir el ambiente pro-inflamatorio en el tejido adiposo y mejorar la homeostasis energética llevada a cabo o regulada desde este. Los mecanismos que provocan estos efectos son aún desconocidos pero podemos descartar la toxicidad mitocondrial ya que no se observan alteraciones en los marcadores de la función mitocondrial en ningún caso.

A pesar de que estos resultados son claros, conviene tener presente que los estudios *in vitro* tienen sus limitaciones. Es difícil concretar las concentraciones reales a las cuales estos fármacos llegan al tejido adiposo de los pacientes. Se ha descrito que la concentración a la que el kaletra aparece intracelularmente en el tejido adiposo es

de aproximadamente 1μM[411], en cambio, si bien queda determinado que los niveles en plasma son de 10μM para el efavirenz[412] o de 20μM para la nevirapina[411] ambos fármacos se acumulan en el tejido adiposo lo cual podría hacer que las concentraciones fueran aún mayores en este tejido. También se debe tener en cuenta que los estudios con preadipocitos en cultivo requieren medios sin suero y este hecho implica que la disponibilidad del fármaco para la célula es mayor debido a la ausencia de unión a proteínas séricas. Por otra parte, estos resultados nos dan una idea sobre la acción de estos fármacos directamente sobre las células adipocitarias lo cual es una importante información para conocer los mecanismos por los cuales se desarrolla la lipodistrofia, pero se debe tener presente que no sería correcto extrapolar directamente los resultados al tejido adiposo de los pacientes ya que el tejido adiposo engloba diferentes tipos celulares además de los adipocitos cuyas respuestas a los tratamientos pueden variar de las observadas. Por otra parte se debe tener en cuenta que los fármacos no se administran de manera individual en la terapia HAART y tal y como se ha comentado anteriormente el efecto sumatorio de los diferentes fármacos administrados conjuntamente, además del efecto de la propia infección por el virus del HIV-1 juegan un papel importante en el desarrollo del síndrome lipodistrófico.

Además de estudiar el efecto de los fármacos directamente sobre adipocitos en cultivo, otro de los objetivos de este estudio de tesis doctoral ha sido describir las alteraciones moleculares que se dan en el tejido adiposo de los pacientes que sufren distintos acontecimientos que varían dependiendo del depósito que se observe como es el caso de la lipohipertrofia que se da en el depósito visceral y la lipoatrofia que sucede en el tejido adiposo subcutáneo. Además de esto, tal y como se ha mencionado con anterioridad, en algunos pacientes se desarrolla lipomatosis en el tejido adiposo subcutáneo en diferentes zonas anatómicas, siendo la zona dorso-cervical la más afectada. Estas acumulaciones de grasa o “buffalo humps” (BH) se dan en el mismo tejido adiposo subcutáneo que normalmente sufre lipoatrofia en los pacientes que sufren HALS. Los motivos que provocan estas diferencias son desconocidos, si bien las diferencias entre el tejido adiposo visceral y el subcutáneo se han descrito para pacientes obesos, aún queda por determinar que sucede durante el síndrome HALS que afecta a los pacientes infectados por el virus HIV-1 que son tratados con terapia HAART. Del mismo modo un estudio molecular del tejido adiposo lipomatoso de la zona dorso-cervical de pacientes con BH es muy interesante para tratar de comprender qué alteraciones pueden explicar el comportamiento lipomatoso de estas zonas. Cabe destacar que en algunas ocasiones se realizan reconstrucciones de las zonas faciales lipoatróficas realizando autotransplantes utilizando la grasa obtenida de las zonas lipomatosas como las BH[355]. En algunos casos estos autotransplantes derivan en lipohipertrofias faciales. Este hecho indica que el tejido adiposo auto-trasplantado mantiene las características de su lugar de origen lipomatoso a pesar de encontrarse en un nuevo ambiente que hasta ese momento resultaba lipoatrófico. Hemos tenido la oportunidad de poder analizar una biopsia de una de estas lipohipertrofias faciales

derivadas de BH, conocidas como síndrome de hámster, para determinar hasta qué punto mantiene estas características moleculares características de zonas lipomatosas.

Uno de los estudios realizados durante esta tesis ha consistido en realizar por primera vez la caracterización comparativa del tejido adiposo hipertrófico visceral respecto al tejido adiposo subcutáneo de pacientes con síndrome lipodistrófico HALS, para determinar si las alteraciones observadas pueden ser la causa del comportamiento opuesto de ambos depósitos. Los estudios realizados anteriormente en el tejido adiposo de pacientes infectados por el virus HIV-1 con lipodistrofia, se habían focalizado en caracterizar el depósito subcutáneo lipoatrófico de los pacientes, probablemente debido a la dificultad de obtener biopsias de tejido omental tanto de pacientes como de individuos control. La mayoría de estos estudios coinciden en señalar la toxicidad mitocondrial como una alteración común en el tejido adiposo lipoatrófico subcutáneo de los pacientes respecto a los controles[413]. En este estudio, hemos descrito como esta toxicidad mitocondrial ocurre de manera similar en el tejido adiposo visceral. A pesar de su diferente comportamiento, la depleción de mtDNA es una tendencia común a ambos tejidos que va acompañada de un descenso de expresión de transcritos para proteínas mitocondriales como la citocromo c o la subunidad IV de la citocromo oxidasa y un aumento de los niveles de proteína mitocondrial, que se entiende como una reacción o un intento de compensación por parte de la célula del daño provocado por la descrita toxicidad mitocondrial, comúnmente asociada a los fármacos NRTI[323]. Estos hechos sugieren que las alteraciones mitocondriales no parecen ser la causa de que el tejido subcutáneo sufra lipoatrofia mientras el visceral presente un comportamiento opuesto. A pesar de esto, podemos apreciar un descenso específico del tejido adiposo lipoatrófico subcutáneo de las proteínas codificadas por el mtDNA, que unido al hecho de que este tejido presente un contenido más bajo de mtDNA, podría implicar una disfunción mitocondrial más aguda en el tejido adiposo subcutáneo que en el visceral. Esta observación concordaría con lo descrito en ratas tratadas con zidovudina, las cuales sufren toxicidad mitocondrial solo en el tejido adiposo subcutáneo[414]. Además un mayor contenido de mtDNA en el tejido adiposo omental podría ayudar a una mejor respuesta compensatoria adaptativa a la alteración mitocondrial (REF). De hecho se ha descrito que la sustitución de los fármacos que resultan más tóxicos a nivel mitocondrial, como los análogos de timidina), por otros que no provocan alteraciones mitocondriales provocan una mejora en la atrofia del tejido adiposo subcutáneo, pero no se advierten signos de mejoría en la hipertrofia del tejido adiposo omental ni en las alteraciones metabólicas[415].

Por otro lado, los estudios anteriormente realizados en el tejido adiposo subcutáneo, tal y como se ha comentado, han descrito un marcado descenso de la expresión de genes marcadores de adipogénesis en el tejido adiposo subcutáneo lipoatrófico de los pacientes. En este estudio reproducimos este resultado con claridad

en el tejido adiposo subcutáneo de los pacientes lipodistróficos, tanto para genes máster de la adipogénesis, como PPARγ, como otros que son dianas de estos (Glut4 o LPL). También se observa, tal y como había sido descrito, una acumulación proteica de SREBP1 no procesada en el tejido adiposo subcutáneo de los pacientes respecto al mismo tejido de los controles sanos. Estos acontecimientos no se reproducen en el tejido adiposo visceral de los pacientes, que no muestran variaciones respecto al tejido adiposo omental de los individuos control, hecho que concuerda con la ausencia de atrofia que se aprecia en este tejido.

Existen diferentes estudios sobre enfermedades metabólicas, como la obesidad, que coinciden en indicar que el nivel de ambiente pro-inflamatorio es una diferencia entre los depósitos visceral y subcutáneo, siendo el tejido adiposo omental el depósito en el que preferencialmente se desarrolla un ambiente más pro-inflamatorio que resulta relevante en el desarrollo de las alteraciones metabólicas asociadas a la obesidad. En individuos sanos, los niveles de algunas citoquinas pro-inflamatorias como IL-6 o MCP-1 es más alto en el tejido adiposo visceral que en el subcutáneo[198]. En nuestro estudio reproducimos estos resultados para los individuos sanos control. También reproducimos el significativo aumento de marcadores relacionados con la inflamación en tejido adiposo subcutáneo respecto a este mismo depósito en los individuos control. Nuestros resultados indican que ambos depósitos estudiados comparten alteraciones respecto a sus correspondientes controles, indicativas de una inducción paralela de un ambiente pro-inflamatorio (como un similar aumento de TNFα o CD68). A pesar de este hecho, existen diferencias entre ambos tejidos adiposos de los pacientes en las alteraciones específicas de algunos marcadores relacionados con la inflamación, lo cual indica una respuesta inflamatoria diferente en ambos depósitos, tanto a la infección por HIV-1 como a los efectos nocivos de los fármacos de la terapia HAART.

Los resultados muestran como el tejido adiposo visceral de los pacientes se da una represión en la expresión de citoquinas típicamente pro-inflamatorias como las interleuquinas 6 y 8 o MCP-1, que se confirma a nivel proteico, acompañado de una represión de la inducción de IL-18 comparado con la inducción que observamos en la expresión de esta citoquina en el tejido adiposo subcutáneo. Estos resultados indican una más moderada reacción pro-inflamatoria en el tejido adiposo visceral de los pacientes que en el tejido adiposo subcutáneo de los mismos. La inducción de citoquinas anti-inflamatorias como IL1RN en el tejido adiposo subcutáneo lipoatrófico puede ser entendida como una reacción al ambiente pro-inflamatorio característico de este tejido, tal y como se ha observado en otras condiciones pro-inflamatorias en este mismo tejido como son la obesidad y la diabetes mellitus tipo II[416].

Ante los resultados observados en estos dos depósitos atrófico vs hipertrófico se puede deducir, de una manera especulativa, que la inducción de las citoquinas pro-

inflamatorias en el tejido adiposo subcutáneo está asociada con los procesos de pérdida de grasa que llevan a la atrofia, mientras en el tejido adiposo visceral, un ambiente inflamatorio más leve puede proteger este depósito de la pérdida de grasa. De hecho, tal y como se ha comentado anteriormente, ha sido descrito que un ambiente inflamatorio en el tejido adiposo es capaz de inhibir procesos adipogénicos (por ejemplo la ya mencionada inhibición de PPARγ por citoquinas pro-inflamatorias). Nuestras observaciones chocan con el conocido papel que juega la inflamación en el tejido adiposo visceral en otras enfermedades metabólicas como la obesidad[417]. En este aspecto, es importante tener en cuenta que la inflamación que se da en el depósito visceral tiene un origen diferente: en la obesidad, la inflamación es provocada por alteraciones intrínsecas en el tejido adiposo causadas por la hipertrofia generada por un balance energético positivo, mientras en la lipodistrofia asociada al tratamiento HAART de pacientes son las agresiones externas como la propia infección por el virus HIV-1 y los efectos dañinos de los fármacos antirretrovirales los que provocan la inflamación en este depósito.

Por otro lado, a pesar de que la aparición de un estado de inflamación local está entre uno de los primeros sucesos descritos durante el desarrollo de la lipodistrofia, incluso antes que la aparición de la lipoatrofia en el tejido adiposos subcutáneo, lógicamente no se puede descartar que la propia lipoatrófia provoque una inducción de las vías pro-inflamatorias en el tejido adiposo subcutáneo, estando el tejido adiposos visceral protegido de esta inducción por su falta de atrofia.

El caso de la lipomatosis es un acontecimiento que presenta un panorama diferente debido a que sucede en el mismo depósito subcutáneo en el que la alteración más común es la lipoatrofia y de hecho sucede al mismo tiempo en el mismo paciente, que puede sufrir ambas alteraciones como parte de su proceso lipodistrófico[312]. Existen diferentes estudios que han descrito las variaciones moleculares que sufre el tejido adiposo subcutáneo lipoatrófico de los pacientes HIV-1 positivos en tratamiento HAART que sufren el síndrome HALS respecto a los controles, tal y como se ha comentado, pero hasta la publicación de nuestro estudio no existía otro que abordara las alteraciones moleculares que se dan en la zona lipomatosa en comparación con las de la zona lipoatrófica subcutánea y tejido adiposo subcutáneo de controles.

Uno de los principales hallazgos de este estudio ha sido la confirmación de que las BHs expresan específicamente UCP1 a diferencia del tejido adiposo subcutáneo de controles y de zonas lipoatróficas. Teniendo en cuenta que la expresión de UCP1 es una característica específica del tejido adiposo marrón[354] su expresión en este tejido lipomatoso indica que existe una alteración en el patrón de diferenciación de los adipocitos de esta zona anatómica. Se ha descrito que la estavudina y la nevirapina son capaces de inducir la diferenciación de adipocitos marrones en cultivo así como de

inducir la expresión de UCP1[418]. En este estudio ya se han presentado resultados que concuerdan con la capacidad de la nevirapina para provocar la diferenciación también de adipocitos blancos en cultivo. De todas formas este hecho no puede explicar que se desarrolle lipomatosis en determinadas zonas anatómicas como en este caso la zona dorso-cervical. Del mismo modo, no existían diferencias en la presencia de estos fármacos en los regímenes de tratamiento HAART de los pacientes estudiados y de hecho existe una publicación previa que descarta una correlación entre los diferentes fármacos de la HAART y la aparición de BHs[419]. Otro de los hechos observados va en sintonía con la expresión de UCP1 y por tanto de obtención de un fenotipo "brown-like" de estos adipocitos presentes en el tejido adiposo de estas acumulaciones lipomatosas, y es la activación de biogénesis mitocondrial (se aprecia un claro aumento de VDAC). En contraste con estos hallazgos, no se observaron en este estudio de las BHs variaciones de UCP3 y PGC1α respecto a los controles. Estos dos genes son típicamente más expresados en el tejido adiposo marrón que en el blanco[241;420]. Este hecho indica que, si bien el tejido adiposo lipomatoso de las BH adquiere ciertas características de tejido adiposo marrón, mantiene otras de tejido adiposo blanco, pudiendo considerarse un tejido adiposo blanco pero "brown-like" o con un fenotipo intermedio.

Como se ha comentado anteriormente, hemos tenido la oportunidad de tener acceso a una muestra de tejido adiposo lipomatosos de una "buffalo hump" que había sido trasplantado a la cara lipoatrófica de un paciente, y tras el trasplante este tejido adiposo había mantenido su tendencia lipomatosa. De este modo, esta muestra nos ha servido para determinar en qué medida se mantenían las características moleculares del tejido adiposo lipomatoso de las "buffalo humps" en los trasplantes autólogos que finalmente derivan en la lipomatosis facial conocida como "síndrome de hámster"[355]. Siendo la expresión de UCP1 un factor clave para determinar el fenotipo "brown-like" de adipocitos de las BHs, se amplió el estudio de marcadores de tejido adiposo marrón respecto a blanco en las "buffalo hump" para poder comparar ambas zonas. En esta ampliación se observó que SIRT3, otro marcador de tejido adiposo marrón[421] presenta un patrón similar a UCP1 en el tejido adiposo lipomatoso dorso-cervical, es decir aparece aumentado en las BHs respecto a las zonas lipoatróficas, y este aumento, tal y como sucede para UCP1 se mantiene una vez esta masa adiposa es auto-trasplantada a la zona facial. Del mismo modo, tal y como se había observado, otros marcadores de tejido adiposo marrón como PGC1α u otros como la 5-deiodinasa, no se ven alterados en estos lipomas. Hecho que también se mantiene una vez trasplantado el tejido adiposo a la zona facial lipoatrófica. Por otra parte en esta ampliación se observó que PRDM16, un recientemente identificado marcador del linaje celular marrón de adipocitos[231], sigue un patrón similar a PGC1α, es decir no se ve alterado. Este hecho va en consonancia con la idea de que los adipocitos de esta zona dorso-cervical adquieran un fenotipo "brown-like" pero los datos obtenidos, como el del marcador PRDM16 parecen descartar que estos adipocitos provengan de células con un linaje de

adipocito marrón. Resulta interesante que una vez auto-trasplantado este tejido adiposo lipomatoso a la zona facial este fenotipo "brown-like" que hemos propuesto para estos adipocitos se mantenga independientemente del ambiente lipoatrófico de su nueva ubicación.

El reciente descubrimiento de depósitos de tejido adiposo marrón activos en adultos humanos precisamente en la zona cervical[301] (ver apartado 3.C.IV. De la introducción general) podría indicar que precursores del linaje de adipocitos marrones puedan estar involucrados en la aparición de la lipomatosis en las BH, por su clara coincidencia anatómica. Aún así, en contraste con esto, es importante tener en cuenta el ya mencionado resultado de la expresión de PRDM16, ya que este marcador no sufre variación significativa en las BHs y la zona facial lipomatosa respecto a los controles de tejido adiposo subcutaneo, del mismo modo que otros marcadores típicos de tejido adiposo marrón como PGC1-α. Una de las hipótesis para explicar este hecho es que la lipomatosis de la zona dorso-cervical afecta al tejido adiposo subcutáneo de esa zona, provocando que adipocitos de linaje blanco sufran una alteración en su fenotipo convirtiéndose en adipocitos de *tipo* marrón o de fenotipo intermedio, pero sin relación con el linaje de adipocitos marrones, mientras parece que los depósitos activos de tejido adiposo marrón detectados en adultos que si provienen de precursores del linaje de adipocitos marrones se encuentran en zonas más interiores[301]. De cualquier modo, resulta evidente que será necesario continuar investigando el origen y el fenotipo de los adipocitos de estos depósitos para aclarar este hecho.

Por otro lado es interesante que la inducción de UCP3 si que se observa en el tejido adiposo subcutáneo lipoatrófico en contraste con los lipomas en la zona dorso-cervical, hecho que en nuestro grupo hemos descrito que está asociado a una inducción de la lipolisis[321] ya que UCP3 tiene una alta sensibilidad a los NEFA[422]. En cambio la no inducción en el tejido lipomatoso adquiere sentido desde el punto de vista de que en este tejido se acumula grasa.

Una de las observaciones de este trabajo es que el tejido adiposo lipoatrófico y el lipomatoso de BHs comparten un descenso en los niveles de mtDNA así como de componentes de la cadena respiratoria, indicando que en ambos se sufre una toxicidad mitocondrial provocada por los fármacos utilizados en la terapia HAART sumado quizá a un efecto de la propia infección por el virus HIV-1. Esta observación concuerda con lo observado en el estudio del tejido adiposo visceral lipohipertrófico con el subcutáneo lipoatrófico. Este hecho indica que se podría descartar la disminución de mtDNA como un hecho que pueda explicar el comportamiento diferencial entre ambos tejidos ya que ambos la sufren. A pesar de este descenso, cabe mencionar, que las proteínas mitocondriales en general se ven sorprendentemente aumentadas respecto a los niveles del tejido adiposo subcutaneo de controles en ambos tejidos adiposos

lipoatrófico y lipomatoso. Este hecho ya se había descrito anteriormente en el tejido adiposo lipoatrófico de pacientes bajo terapia HAART[323], así como he comentado que hemos demostrado en estos pacientes a nivel del depósito adiposo visceral y del mismo modo que explicaba para este último, ha sido interpretado como un mecanismo de compensación en un intento por parte de las mitocondrias de contrarrestar los bajos niveles de mtDNA que sufren debido a la toxicidad mitocondrial de algunos fármacos, sobre todo los NRTI.

A nivel adipogénico se observan algunos cambios que podrían ser una de las claves que podrían explicar el comportamiento opuesto de estos depósitos adiposos subcutáneos. A diferencia del tejido adiposo subcutáneo lipoatrófico, y tal como ocurría en el tejido adiposo visceral hipertrófico de los pacientes, las BHs no presentan una represión del gen master de la adipogénesis PPARγ respecto a los controles. Además en los BHs, del mismo modo que en el tejido adiposo subcutáneo de controles, no se observa la inducción de la expresión del factor anti-adipogénico Pref-1 que se observa en tejido adiposo lipoatrófico subcutáneo. Estas observaciones resultan coherentes con el aumento de masa adiposa en las "buffalo humps" respecto al tejido adiposo subcutáneo lipoatrófico. Del mismo modo, los genes relacionados con la adquisición de lípidos por parte de los adipocitos como la LPL o adiponectina, que son dianas de PPARγ[423;424], también están aumentados en lipomas en comparación con el tejido adiposo subcutáneo lipoatrófico. Por otra parte, en este mismo ámbito adipogénico, tanto nuestro grupo como otros habían observado un aumento a nivel proteico de SREBP1 en el tejido adiposo subcutáneo lipoatrófico de los pacientes que sufrían HALS. Recordemos que SREBP1 es una proteína que sufre maduración post-transcripcional y por lo tanto, este acontecimiento se ha relacionado con la acumulación de SREBP1 procesada de manera defectuosa[322]. En este estudio se reproduce este resultado para el tejido adiposo subcutáneo lipoatrófico de los pacientes, pero este aumento proteico de SREBP1 no se aprecia en el tejido adiposo lipomatoso.

Continuando en el ámbito adipogénico, es interesante como de nuevo se observa que al ser trasplantado a la zona facial, este tejido adiposo de BH mantiene las mismas características adipogénicas que cuando se encontraba en la zona lipomatosa dorso-cervical. Este hecho es importante ya que la región a la que se trasplanta el tejido lipomatoso, es la zona facial, una región altamente lipoatrófica durante el HALS debido a lo cual se hace necesario el trasplante. El hecho de que el ambiente de la nueva localización sea lipoatrófico no parece afectar al tejido lipomatoso trasplantado indicando que el fenotipo que ha adquirido parece ser permanente y no reacciona a los diversos estímulos lipoatróficos que puede recibir en su nueva localización anatómica.

Resulta un punto interesante comentar que las tiazolidindionas, como la rosiglitazona, han sido propuestas como una posible terapia para el tratamiento de la lipodistrofia así como también para la resistencia a la insulina asociada a esta ya que estos fármacos son capaces de incrementar la masa de grasa subcutánea y reducen la resistencia a insulina en los pacientes con diabetes mellitus tipo II[425]. A pesar de esta posibilidad es importante tener en cuenta los resultados obtenidos durante esta tesis, ya que, si bien es posible que se obtuvieran efectos beneficiosos, los pacientes tratados correrían el riesgo de desarrollar lipomatosis o que estas se acentuaran. De hecho ha sido descrito el caso de un paciente bajo terapia HAART tratado con tiazolidindionas que ha desarrollado una lipomatosis multiple[426].

Uno de los acontecimientos más descritos en el tejido adiposo subcutáneo de los pacientes infectados por el HIV-1, tratados con HAART y que sufren HALS es un ambiente local inflamatorio[314], en el que destacan el aumento de expresión de TNFα y otras citoquinas pro-inflamatorias. De hecho, muchas de las alteraciones de expresión génica que se sufren en este tejido, como una represión de la expresión de PPARγ y por lo tanto de sus genes diana relacionados con la adipogénesis, podrían ser explicadas desde el punto de vista de la represión que provocan los altos niveles de TNFα en su expresión[314]. En este estudio también observamos este aumento de marcadores de inflamación en el tejido adiposo lipoatrófico de los pacientes respecto a los controles, pero en contraste con esto, el tejido adiposo lipomatoso de los BHs no ve alterada su expresión de TNFα o β-2-microglobulina, una diana directa de citoquinas pro-inflamatorias. Este hecho va de acuerdo con los niveles normales de marcadores de adipogénesis en el tejido adiposo lipomatoso, ya que los genes adipogénicos no se verían afectados por los altos niveles de TNFα, entre otros. De todos modos resulta sorprendente que tratándose las gibas de búfalo de tejido adiposo subcutáneo, no se sufra en esta zona anatómica el efecto de inflamación local provocado tanto por la terapia HAART como por la propia infección por parte del virus HIV-1. Aún así, y desconociendo el motivo por el que este hecho no es común a todo el depósito de tejido adiposo subcutáneo independientemente de la región en la que se encuentre, esta diferencia en la inflamación local aporta una de las diferencias que podrían explicar, junto a otras como la diferencia en la represión de genes adipogénicos, el porqué de este comportamiento opuesto en el que una zona sufre acumulación mientras otra sufre pérdida de masa adiposa. Igualmente las observaciones microscópicas de los tejidos adiposos subcutáneos con diferente comportamiento indican una mayor infiltración de macrófagos en el tejido adiposo subcutáneo lipoatrófico[318]. Se debe tener en cuenta que esta infiltración puede contribuir al estado de inflamación local. Siguiendo en esta línea y de acuerdo con este hecho, en el tejido adiposo subcutáneo lipoatrófico se observa una inducción de CD68, marcador de infiltración de macrófagos, que no se aprecia en el tejido adiposo lipomatosos de las BHs. Cuando este tejido es trasplantado a la zona facial mantiene, nuevamente, este estado no inflamado, apuntando de nuevo a un fenotipo alterado adquirido en la zona

lipomatosa que es permanente ya que no varía al ser ubicado en un ambiente diferente.

Otro de los puntos que nos interesaba estudiar en este trabajo era la proliferación de los adipocitos en ambos tejidos adiposos subcutáneos. Los resultados para PCNA un marcador de proliferación celular cuya expresión se ve altamente inducida en el tejido adiposo lipomatoso respecto a las zonas lipoatróficas, indican que existe una mayor proliferación en las BHs. Esta capacidad proliferativa es consecuente con la adquisición de un fenotipo parecido al del adipocito marrón con mayor capacidad proliferativa y diferenciativa, tal y como sucedía con la expresión de genes como UCP1 o SIRT3, por parte de los adipocitos de la zona dorso-cervical lipomatosa. Resulta lógico pensar que esta capacidad proliferativa se mantiene en el tejido adiposo lipomatoso auto-trasplantado a la zona facial, aunque este hecho no sucede en todos los casos en los que se realiza esta cirugía.

Por tanto, las gibas de búfalo son alteraciones muy específicas dentro de las alteraciones adiposas que sufren los pacientes HIV-1 con síndrome lipodistrófico y son varios los cambios a nivel molecular relacionadas con esta lipomatosis, como son: la adquisición de un fenotipo "brown-like" y la inducción de la proliferación. Además, y en contraste con el tejido adiposo subcutáneo de estos pacientes, no ven alterada su capacidad adipogénica así como su estado inflamatorio. Estas alteraciones se mantienen y son permanentes en los casos en los que se desarrolla el síndrome de "hámster" y están relacionadas con una adquisición de un fenotipo intermedio entre el adipocito blanco y el marrón. Así, una vez este tejido es cambiado a una localización con un ambiente lipoatrófico, el tejido adiposo lipomatoso mantiene las características que se habían descrito en su ambiente de origen. Es necesario mencionar que el síndrome "hámster" no se desarrolla en todos los casos en los que se da el autotrasplante de tejido adiposo lipomatoso dorso-cervical a la zona facial. La razón por la que en unos pacientes se mantiene la capacidad proliferativa y en otros no, es desconocida. Debido a la variabilidad que hemos observado en la expresión génica de los diferentes pacientes con BHs, es posible que solo en aquellos en los que el fenotipo adquirido haya sido más parecido al fenotipo de adipocito marrón, con mayor capacidad proliferativa, sean los que presentan una mayor probabilidad de desarrollar este síndrome de "hámster". Es necesario realizar más estudios de este tipo de lipomatosis faciales para determinar si esta hipótesis resulta cierta. Si se confirma este hecho, estudios de expresión génica de los fenotipos de los adipocitos de las BHs podrían determinar si sería adecuado realizar un auto-trasplante o se corre el riesgo de sufrir el síndrome de "hámster".

Resulta interesante que a pesar de que el tejido adiposo lipomatoso de las "buffalo hump" pertenece al tejido adiposo subcutáneo a diferencia del tejido adiposo visceral de los pacientes, en ambos casos el crecimiento del depósito se asocia a una

deleción mitocondrial similar a la del tejido adiposo subcutáneo lipoatrófico; así como una expresión de marcadores de adipogénesis preservada respecto a la grasa lipoatrófica y una ausencia de inducción de un marcado estado inflamatorio. Estas observaciones, apoyan la idea de que la inflamación esté estrechamente ligada al estado lipoatrófico del tejido adiposo subcutáneo en los pacientes tratados con terapia HAART. A pesar de esto, es necesario tener en cuenta las limitaciones de estos estudios que dependen siempre del número de biopsias disponibles, cuyo bajo número nos impide clarificar entre otras cosas el papel que juegan los diferentes patrones de administración de los diferentes fármacos, que como se ha mostrado en los estudios comparativos realizados, difieren importantemente en los efectos sobre el tejido adiposo. Así mismo, siendo las biopsias de tejido adiposo visceral las más complicadas de lograr, para lograr un entendimiento mayor de las diferencias entre los dos depósitos visceral y subcutáneo, resultaría más que interesante estudiar muestras de ambos tejidos, e incluso si existiera la posibilidad, incluir una muestra de una zona lipomatosa, pertenecientes a los mismos individuos.

El síndrome lipodistrófico, además de incluir la lipodistrofia generalizada acompañada de la adiposidad central y la lipomatosis, engloba diferentes disfunciones metabólicas. Estas alteraciones están asociadas con la redistribución de tejido adiposo que sufren los pacientes y quedan enmarcadas dentro del conocido como síndrome metabólico que principalmente incluye dislipidemias, hipertensión arterial, resistencia a la insulina y diabetes mellitus. Resulta complejo determinar el origen de estas disfunciones metabólicas. Ante el creciente interés suscitado por los FGFs endocrinos por su relación con la homeostasis metabólica, especialmente FGF19 y FGF21, durante esta tesis se han estudiado ya que es de alto interés identificar agentes capaces de explicar estas alteraciones para comprender sus orígenes y su desarrollo, así como determinar hasta qué punto pueden ser útiles a la hora de prevenir el síndrome metabólico.

El primer hecho que observamos en este ámbito fue que los niveles de FGF21 estaban aumentados en el suero de los pacientes infectados por el virus HIV-1. De hecho los niveles aparecen aumentados en todos los pacientes infectados, pero van incrementando a medida que pasamos de pacientes infectados sin tratamiento HAART, a tratados que no presentan síndrome lipodistrófico, para llegar a su máximo nivel de aumento en los pacientes infectados tratados con síndrome lipodistrófico. Esta observación parece ir de acuerdo con el hecho de que los pacientes tratados con terapia HAART que sufren HALS presentan las mayores alteraciones metabólicas. Por otra parte, resulta significativo que tal y como se había observado en el tejido adiposo con acontecimientos como la inflamación o la alteración inicial del metabolismo lipídico, los pacientes sin tratamiento, es decir los “naives”[321], sufren ya una alteración que después se acentúa o se acelera a medida que estos pacientes comienzan el tratamiento. De hecho entre los pacientes tratados no se observó ninguna correlación

significativa entre los valores anormales de FGF21 y duración de tratamiento con los diferentes tipos de fármacos. Si bien, los PIs han sido los que tradicionalmente más se han asociado con la aparición de disfunciones metabólicas o síndrome metabólico en este caso no apreciamos esta relación.

Los niveles de FGF21 observados en suero se correlacionaban fuertemente con indicadores de alteraciones en la distribución de tejido adiposo y por tanto de lipodistrofia (como pueden ser el ratio torácico-apendicular) señalando una relación entre esta y el aumento de FGF21 en suero. La falta de relación de FGF21 con los niveles de masa de grasa o con el BMI en contraste con lo que se había estudiado con pacientes obesos[400] es posiblemente consecuencia del tipo de población de estudio de este trabajo ya que implica un grupo de personas que se mueven en un estrecho rango de BMI que no incluye pacientes obesos.

Uno de los hechos más interesantes apreciados en este estudio es la correlación de los niveles de FGF21 en suero con indicadores de sensibilidad a insulina o de síndrome metabólico, como son HOMA-R, glucosa o colesterol LDL. Esto concuerda perfectamente con lo observado hasta el momento en estudios con obesos en los que se correlacionan los altos niveles de FGF21 con el síndrome metabólico[427]. Resulta paradójico como en obesos y pacientes con síndrome metabólico los niveles de FGF21 aparecen aumentados cuando todos los estudios realizados con modelos murinos indican que FGF21 es un agente anti-diabético. Lógicamente la debatida teoría de la resistencia a FGF21 que ha sido propuesta para la obesidad[402] sería también válida para estos pacientes ya que a pesar de tratarse de dos anomalías distintas comparten el aumento de FGF21 relacionado a síndrome metabólico.

El hecho de que los pacientes “naive” sufran ya un aumento en los niveles de FGF21 que va aumentando con el tratamiento y con la aparición de la lipodistrofia, sumado a su fuerte correlación con las alteraciones metabólicas que engloban el síndrome metabólico que sufren estos pacientes, es una interesante aportación que abre una puerta a la opción de que FGF21 pueda ser estudiado como un predictor de estas futuras alteraciones metabólicas.

Por otro lado, los estudios más recientes sitúan el hígado como el órgano responsable de los niveles en suero de FGF21[362], pero algunos estudios indican que FGF21 también se expresa en el tejido adiposo humano[400]. En el caso de los pacientes analizados en este estudio, este acontecimiento resultaba interesante de investigar ya que siendo así, la expresión de FGF21 en el tejido adiposo lipoatrófico y en el lipohipertrófico visceral colaborarían con el hígado en el aumento de los niveles de este FGF endocrino en suero. Nuestras observaciones nos llevaron a concluir que la expresión de FGF21 en tejido adiposo humano es prácticamente 100 veces inferior a la del hígado y a menudo indetectable, y que por lo tanto es la expresión en el hígado la que determinaría un aumento en suero de FGF21 como el observado. Este hecho

adquiere aún más sentido si tenemos en cuenta que los activadores de PPARγ como la rosiglitazona son capaces de activar la expresión de FGF21 en adipocitos en cultivo[427], y los pacientes infectados por HIV-1 bajo terapia HAART con lipodistrofia sufren una disminución de la expresión precisamente de PPARγ.

En relación al hígado, cabe destacar que los niveles en suero de FGF21 se correlacionaban positivamente con los marcadores de daño hepático AST, ALT y sobre todo con GGT. Este hecho había sido descrito para pacientes con alteraciones en la tolerancia a la glucosa[428]. En nuestro estudio se habían descartado pacientes con cirrosis, con problemas de alcoholismo o con obstrucciones biliares, además retirando los pacientes con doble infección HIV-1 y hepatitis C las correlaciones se mantienen. Esto indica que estos marcadores de daño hepático probablemente estén asociados a esteatosis hepática no alcohólica. De hecho algunos estudios han descrito que los niveles de FGF21 están aumentados en suero en pacientes con esteatosis hepática no alcoholica[429]. En modelos de roedores el flujo de lípidos al hígado aparece como un inductor del aumento de la expresión de FGF21 en este órgano[394;396]. En esta línea existen múltiples estudios que relacionan la lipodistrofia de los pacientes infectados por HIV-1 bajo terapia HAART con la esteatosis hepática[430]. La hiperlipidemia es un hecho común durante la lipodistrofia y este aumento de disponibilidad lipídica en el hígado podría provocar el aumento en la expresión de FGF21 vía activación de PPARα. Este hecho está altamente relacionado con lo que se ha comentado en la introducción (apartado 4.F.) presentando la lipotoxicidad como mecanismo por el cual un aumento de disponibilidad de ácidos grasos que se acumulan ectópicamente en tejidos como el hígado puede provocar una disfunción en estos, como ocurriría en este caso. Aunque, desde luego, no se puede descartar la idea de que exista un agente desconocido aún, inducido por la infección de HIV-1 y el tratamiento HAART que lleva a la lipodistrofia que provoque la inducción de FGF21 en el hígado y que precisamente sea este aumento de FGF21 en suero lo que provoque la lipólisis en el tejido adiposo y que de este modo esta lipólisis sea la que provoque un flujo aumentado de lípidos al hígado. Esta es otra hipótesis plausible, pero es importante tener en cuenta que la acción lipolítica de FGF21 en la que está basada, al ser tan reciente la irrupción de este agente endocrino como regulador de la homeostasis metabólica, se encuentra en pleno debate científico y no existe un acuerdo sobre los efectos reales de FGF21 a este nivel.

Así mismo, también son recientes los estudios que describen FGF19 como otro de los miembros de la superfamilia de FGFs endocrinos que toma parte en la regulación de la homeostasis metabólica. Resultaba muy atractivo estudiar el comportamiento en los niveles de este FGF19 en los mismos grupos de pacientes en los que se había estudiado FGF21, ya que ambos, tal y como se ha comentado en la introducción, parecen jugar un papel determinante en la regulación de la glucemia, la lipidemia y la resistencia a insulina, que por otra parte son precisamente los procesos

que se ven alterados en los pacientes que sufren lipodistrofia asociada a la terapia HAART. Por eso los niveles que presentan los pacientes de FGF19 sumado a lo que ya sabíamos sobre FGF21, y que se reprodujo en este segundo estudio, puede ser de mucha utilidad para poder explicar las acciones a las que se asocian estos dos agentes.

En este estudio observamos como los niveles en suero de FGF19, al contrario de lo que sucedía para FGF21, están reducidos en los pacientes infectados por el virus HIV-1. De nuevo, los pacientes "naive" ya muestran un descenso significativo de sus niveles de FGF19 en suero respecto a los controles. Este descenso se ve aumentado ante la presencia de los fármacos de terapia HAART y presenta el punto más bajo con la aparición de la lipodistrofia asociada a estos. Dentro de la variedad de fármacos de la terapia HAART este estudio demuestra como los niveles de FGF19 se correlacionan negativamente con la acumulación de meses recibiendo fármacos NRTIs, particularmente la estavudina, y con el NNRTI efavirenz. Resulta interesante que la estavudina sea uno de los fármacos más asociados con las alteraciones del tejido adiposo y la lipodistrofia debido a su alta toxicidad mitocondrial[323], ya que tal y como observamos en los niveles de FGF19, se da un descenso progresivo siendo el punto más bajo de los niveles en suero en el grupo de pacientes lipodistróficos. A pesar de esto, y en contraste con FGF21, no hemos observado una correlación negativa significativa entre los típicos marcadores de lipodistrofia y los niveles en suero de FGF19 aunque sí una clara tendencia a que los niveles en suero de este FGF endocrino sean inferiores en los pacientes que muestran marcadores indicadores de lipodistrofia. Por otra parte, el efavirenz no se había asociado con la aparición de síndrome metabólico o de lipodistrofia y de esta manera se había asumido que era un fármaco relativamente "benigno" para el tejido adiposo. Pero tal y como se ha presentado en este estudio de tesis, nuestras investigaciones *in vitro* muestran una alta capacidad nociva de este fármaco en los adipocitos en cultivo. El efavirenz ha resultado ser un potente inhibidor de la adipogénesis, que además reprime la secreción de adipoquinas antidiabéticas como la adiponectina y aumenta la secreción de citoquinas pro-inflamatorias generando una ambiente pro-inflamatorio. La extrapolación de estos resultados al tejido adiposo humano de los pacientes resulta arriesgada y altamente especulativa. De hecho existe un reciente estudio que refuerza esta "nueva" visión del efavirenz, se trata de un estudio clínico aleatorio de fármacos que indica significativamente que la lipodistrofia en estos pacientes es más común con el efavirenz que con los PIs cuando se combinan ambos con estavudina o zidovudina[83]. Viendo esto en conjunto, la correlación observada entre meses acumulados consumiendo efavirenz y niveles en suero de FGF19 adquiriría sentido siempre y cuando se asuma que el efavirenz afecta al tejido adiposo de los pacientes, siendo uno de los fármacos que potenciarían la lipodistrofia de manera que del mismo modo potenciaría disfunciones metabólicas que derivarían en la alteración en los niveles de FGF19.

Los conocimientos actuales sobre la regulación de la producción y secreción de FGF19 actualmente son limitados, por lo tanto resulta complicado y especulativo explicar este descenso que observamos en los pacientes infectados. Tal y como se ha explicado en la introducción (apartado 5.B.), el ileum es el principal productor de este FGF endocrino, inducido por los ácidos biliares. Existe un estudio publicado que describe los niveles de ácidos biliares en pacientes infectados por el HIV-1[431]. En este estudio se presentan unos resultados con una alta variabilidad inter-individual de los pacientes, observándose aumentos de ácido litocolico y taurocolico en algunos y descensos como el del ácido quenodesoxicólico en otros. Lógicamente serán necesarios futuros estudios para determinar la implicación de los niveles de ácidos biliares en este descenso de FGF19 en suero que se aprecia en los pacientes infectados.

Por otra parte los niveles de FGF19 se correlacionan negativamente con parámetros indicativos de resistencia a insulina, como el HOMA-R. Este hecho tiene sentido desde el punto de vista del papel que se ha propuesto para FGF19 como promotor de la sensibilidad a la insulina y la absorción de glucosa en los tejidos diana como el tejido adiposo[362]. Como se ha explicado en la introducción, esta proposición del papel de FGF19 está basada en estudios en modelos animales y cultivos. El estudio transversal que hemos realizado conlleva ciertas limitaciones, y no se pueden establecer ciertas relaciones de causalidad. A pesar de esto podemos decir que es probable que los bajos niveles de FGF19 en suero descritos para los pacientes infectados contribuyan a las alteraciones metabólicas que normalmente sufren estos pacientes como la hiperglucemia y la resistencia a insulina. Tal y como se comentaba en la introducción, se ha propuesto que el tratamiento farmacológico de modelos de roedores con FGF19 estimula algunos efectos que normalmente provoca la insulina en el hígado[362]. Esta observación iría de acuerdo con los resultados que hemos obtenido para los pacientes, en los que los bajos niveles de FGF19 disminuirían estos efectos.

Uno de los puntos más interesantes de este estudio ha sido la descripción de la reducción en la expresión del receptor FGFR1 y del co-receptor β-Klotho en el tejido adiposo de los pacientes infectados respecto a los controles. De hecho, este estudio ha sido el primero en determinar una alteración de la expresión génica de los componentes de respuesta a FGF endocrinos en humanos. Tal y como se ha comentado anteriormente ambos mediadores son la base de la respuesta en el tejido adiposo a FGF19 y FGF21, por lo tanto este resultado apoya la idea de la existencia de una disminuida capacidad de respuesta a estos FGF endocrinos. Este hecho va de acuerdo con la ya comentada propuesta de la existencia de una resistencia a FGF21 que se había propuesto en un estudio con ratones obesos en el que del mismo modo se observaba un descenso de la expresión de los receptores FGF y de β-Klotho en el tejido adiposo blanco de los roedores[402]. Hay que tener en cuenta que como hemos demostrado, los pacientes infectados presentan unos altos niveles de FGF21 en suero

comparados con los controles, pero parece que estos altos niveles no son capaces de llevar a cabo las acciones benignas sobre el metabolismo de la glucosa o la resistencia a la insulina que se han descrito ya que tal y como observamos, los marcadores de alteraciones metabólicas, así como los de lipodistrofia se correlacionan con estos altos niveles de FGF21. Por lo tanto una posible resistencia a este FGF adquiriría cierto sentido y aún más con esta última observación de un descenso de la expresión de los mediadores de la respuesta de FGF21 y FGF19 en el tejido adiposo de los pacientes. El panorama para FGF19 es ligeramente diferente ya que, en contraste con FGF21, se aprecia un descenso de sus niveles en suero en los pacientes infectados en comparación con los controles. Este hecho acompañado por el descrito descenso de la expresión de los componentes encargados de mediar su acción en el tejido adiposo explicaría la represión de sus descritos beneficiosos efectos metabólicos sobre el tejido adiposo, que parece ser una de sus importantes dianas.

Como ya se ha mencionado, el tema de los FGFs y sus receptores está en plena ebullición científica en estos momentos. Esto implica que la información que se puede obtener es aún limitada y no faltan los debates respecto a diferentes ideas u observaciones. Los mecanismos de regulación de los receptores de FGFs y β-Klotho son a día de hoy desconocido. Podemos decir, basándonos en nuestras observaciones, que la simple presencia de la infección por el virus HIV-1 provoca una represión en la expresión de estos mediadores de la respuesta de FGF endocrinos. Antes habíamos descrito que la propia infección por HIV-1 ya es capaz de generar alteraciones en el metabolismo lipídico en el tejido adiposo o de provocar un estado pro-inflamatorio en el mismo[321]. Entre los hechos que se dan durante la infección de los pacientes por este virus, sin aún recibir terapia HAART, habíamos descrito una represión en la expresión de PPARγ en el tejido adiposo. Este descenso parece ser una consecuencia del estado inflamatorio que se provoca en el tejido adiposo, ya que algunas señales pro-inflamatorias como TNFα, son capaces de inhibir la expresión de PPARγ, así como algunas de las proteínas secretadas por el propio virus como Vpr[321]. Se ha descrito que β-Klotho está fuertemente regulado por PPARγ[432]. Este hecho explicaría un descenso en la expresión de β-Klotho en los pacientes infectados respecto a los controles debido a un anormal funcionamiento de su regulador PPARγ. Recientemente, además, nuestro grupo ha descrito como TNFα es capaz de inhibir la expresión de β-Klotho[433].

Viendo los resultados para ambos FGFs y los componentes de sus receptores en el tejido adiposo de manera global podemos decir que probablemente la sensibilidad a FGF19 y FGF21 esté reducida en los pacientes infectados por el virus HIV-1, atenuando los efectos beneficiosos metabólicos que se han descrito para estos FGFs endocrinos. Este hecho contribuiría a las alteraciones metabólicas que sufren estos pacientes. Es interesante que en ratones sin sensibilidad para FGF21, dosis farmacológicas del mismo sean aún capaces de provocar efectos beneficiosos[402]. De esta manera parece importante seguir investigando estos dos agentes endocrinos FGF21 y FGF19 ya que

futuras investigaciones podrían determinar que su utilización farmacológica provoque mejorías en estos pacientes que sufren síndrome metabólico y lipodistrofia, así como en otras alteraciones con patrones similares.

CONCLUSIONES

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- En adipocitos en cultivo el Efavirenz es un fármaco que provoca con más potencia que el Kaletra (Lopinavit/Ritonavir): una inhibición de la diferenciación, un descenso en la liberación de adipoquinas anti-diabéticas (como adiponectina) y un aumento de liberación de citoquinas pro-inflamatorias.

- La Nevirapina no inhibe la adipogénesis e incluso la aumenta a determinadas concentraciones en adipocitos en cultivo. Además provoca un descenso en la expresión y liberación de citoquinas pro-inflamatorias.

- La caracterización molecular de la lipomatosis dorso-cervical "buffalo hump" de pacientes HIV-1 en terapia HAART con lipodistrofia, indica que se trata de una alteración específica, y diferente a la lipoatrofia dentro de las alteraciones adiposas que sufren los pacientes. Son varios los cambios específicos relacionados con esta lipomatosis, como son: la adquisición de un fenotipo "brown-like" y la inducción de la proliferación. Además, y en contraste con el tejido adiposo subcutáneo de estos pacientes, el tejido adiposo de "buffalo hump" no ve alterada su capacidad adipogénica así como tampoco su estado inflamatorio.

- El fenotipo y las características moleculares adquiridas por los adipocitos del tejido adiposo lipomatoso de la zona dorso-cervical se mantienen cuando este tejido es auto-trasplantado a la zona lipoatrófica facial en un caso estudiado en que el paciente ha desarrollado el síndrome "hámster".

- El patrón de alteraciones en el tejido adiposo visceral y tejido adiposo subcutáneo de pacientes-1 en terapia HAART con lipodistrofia es opuesto en cuanto a la adipogénesis pero similar en relación a la toxicidad mitocondrial. Aunque ambos presentan alteraciones de signo pro-inflamatorio, el tejido adiposo visceral presenta una más moderada inducción de estas.

- Los pacientes infectados por el HIV-1 sufren un aumento en suero de los niveles de FGF21 respecto a individuos control que se ve incrementado a medida que los pacientes comienzan a ser tratados y desarrollan lipodistrofia. Este aumento se correlaciona positivamente con marcadores de resistencia a insulina y síndrome metabólico, así como con marcadores de daño hepático.

- Los pacientes infectados por el HIV-1 con y sin tratamiento muestran un descenso de los niveles de FGF19 en suero respecto a individuos control. Este descenso se correlaciona negativamente con marcadores de resistencia a insulina, así como con los meses acumulados tomando fármacos NRTIs, particularmente Estavudina y el NNRTI Efavirenz.

- En el tejido adiposo de los pacientes infectados por HIV-1, se observa un descenso en la expresión génica de los receptores FGFR1 y β-Klotho, que son los mediadores de la respuesta a los FGF endocrinos en este tejido.

- En resumen, los pacientes infectados por el HIV-1 muestran una marcada alteración en el sistema de síntesis y respuesta fisiológica de los FGFs hormonales, lo que podría tener relación con las alteraciones metabólicas presentes en estos pacientes.

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ARTÍCULOS

Differential effects of Efavirenz and Lopinavir/Ritonavir on human adipocyte differentiation, gene expression and release of adipokines and pro-inflammatory cytokines

Revista: Current HIV Research. 2010 Oct; 8(7):545-53.

PMID: 21073442

Título: Efectos diferenciales del Efavirenz y Lopinavir/Ritonavir en la diferenciación, expresión génica y secreción de adipoquinas y citoquinas pro-inflamatorias de adipocitos humanos

En este estudio se ha llevado a cabo una comparación entre los efectos del efavirenz (EFV) y lopinavir/ritonavir (LPV/r a relación 4:1) en adipocitos humanos en cultivo. Se trataron preadipocitos humanos con EFV o LPV/r durante o tras la diferenciación. Se midieron la adquisición de morfología de adipocito, la expresión de genes relacionados con la toxicidad mitocondrial, adipogénesis e inflamación y la secreción de adipoquinas y citoquinas. Los resultados indican que tanto EFV como LPV/r reprimen la diferenciación adipocitaria viéndose un descenso en los transcritos para genes relacionados con adipogénesis como adiponectina, lipoproteína lipasa o leptina y genes máster de la adipogénesis como PPARγ y C/EBPα. Los efectos son más acusados con EFV que con LPV/r. Ambos inducen los niveles de mRNA de MCP1, pero el efecto era mayor en EFV que en LPV/r. Del mismo modo se ve una mayor secreción de citoquinas pro-inflamatorias y otras moléculas relacionadas con la inflamación como IL6 y 8, MCP1 o PAI-1 en adipocitos tratados con EFV que en los tratados con LPV/r. Los niveles de secreción de adiponectina y leptina resultan reducidos por ambos fármacos siendo reducidos más fuertemente por EFV. Ninguno de los fármacos alteró los niveles mitocondriales de DNA, los transcritos que codifican para proteínas mitocondriales o la secreción de lactato por los adipocitos. En adipocitos ya diferenciados el EFV, a diferencia de LPV/r, provoca una reducción significativa en la expresión de PPARγ y adiponectina. Concluimos que ambos fármacos reprimen la adipogénesis humana, reducen la secreción de adipoquinas y aumentan la expresión y secreción de citoquinas relacionadas con la inflamación, pero estos efectos siempre son mayores con el EFV.

Differential Effects of Efavirenz and Lopinavir/Ritonavir on Human Adipocyte Differentiation, Gene Expression and Release of Adipokines and Pro-Inflammatory Cytokines

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Abstract: In the present study, a comparative assessment of the effects of efavirenz (EFV) and lopinavir/ritonavir (LPV/r; 4:1) on human adipocytes in culture has been performed. Human pre-adipocytes were treated with EFV or LPV/r during or after adipogenic differentiation. Acquisition of adipocyte morphology, expression of gene markers of mitochondrial toxicity, adipogenesis and inflammation, and release of adipokines and cytokines to the medium were measured. Results indicated that EFV and LPV/r impaired adipocyte differentiation in association with a reduction in transcript levels for adipogenic differentiation genes (adiponectin, lipoprotein lipase, leptin) and master regulators of adipogenesis (PPARγ, C/EBPα). The effects were greater with EFV than LPV/r. Both LPV/r and EFV induced increases in monocyte-chemoattactant protein-1 (MCP-1) mRNA levels, but the effect was greater with EFV. Similarly, the release of pro-inflammatory cytokines and other inflammation-related molecules (interleukins 6 and 8, MCP-1, PAI-1) was enhanced to a much higher degree by EFV than by LPV/r. Adiponectin and leptin release by adipocytes was reduced by both drugs, although to a higher extent by EFV. Neither drug affected mitochondrial DNA levels, transcripts encoding mitochondrial proteins or lactate release by adipocytes. In previously differentiated adipocytes, EFV caused a significant reduction in PPARγ and adiponectin expression, whereas LPV/r did not. We conclude that both EFV and LPV/r impair human adipogenesis, reduce adipokine release and increase the expression and release of inflammation-related cytokines, but the overall effects are greater with EFV. These findings may have implications for the pathogenesis of HIV-1-associated lipodystrophy and the development of HIV-1 therapies.

Keywords: Adipocyte, antiretroviral, efavirenz, lipodystrophy, lopinavir, protease inhibitor.

INTRODUCTION

Alterations in systemic metabolism reminiscent of the metabolic syndrome as well as lipodystrophy, which is characterized by peripheral lipoatrophy, visceral obesity and, in some cases, lipomatosis, are common disturbances among HIV-1-infected patients undergoing highly active antiretroviral therapy (HAART). It is thought that these alterations cannot be attributed to a single antiretroviral drug or a unique drug family, but instead reflect a complex interplay of the drug combinations in HAART and possibly to the underlying HIV-1 infection [1]. HAART regimes containing thymidine-analog reverse transcriptase inhibitors, such as stavudine or zidovudine, are considered particularly likely to elicit some of these adverse metabolic effects, especially peripheral lipoatrophy. Protease inhibitors are also thought to have secondary metabolic effects in patients and to have metabolic syndrome-related effects, such as insulin resistance or dyslipidemia.

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Efavirenz (EFV) and lopinavir/ritonavir (LPV/r) are drugs of choice for initial combination of antiretroviral HIV-1 therapy. EFV is a non-nucleoside inhibitor of the HIV-1 reverse transcriptase. Although its potential secondary effects on adipose tissue function and metabolism are poorly understood, this drug has not traditionally been associated with the appearance of lipodystrophy and is generally perceived as associated with a lower risk for metabolic and adipose abnormalities than protease inhibitors. However, a clinical trial has shown that EFV could have greater potential to cause lipoatrophy than LPV/r [2], and an extensive randomized study (ACTG A5142) recently reported that lipoatrophy was more common with EFA than LPV/r when combined with stavudine or zidovudine [3].

The effects of the individual components of the LPV/r combination on adipose cells have been studied *in vitro*. In mouse adipogenic cell lines, ritonavir, and to a lesser extent, lopinavir, impairs adipocyte differentiation [4, 5, 6] and induces the expression of inflammatory cytokines [7]. In human cells, several studies have indicated that ritonavir reduces adipocyte differentiation and induces the expression of pro-inflammatory cytokines [8, 9, 10, 11]. A study by Vernochet *et al*. [11] comparing the effects of ritonavir and lopinavir indicated that the effects of ritonavir were more deleterious. However, few studies have reported the effects of LPV/r combination therapy on human adipocytes, and a

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single report indicates that treatment with ritonavir plus lopinavir induced a greater reduction in glucose uptake by human adipocytes than ritonavir alone [12]. Studies on the effects of EFV on adipocytes *in vitro* are scarce. One study using the 3T3-L1 mouse white adipocyte cell line reported that EFV reduces the capacity of pre-adipocytes to accumulate triglycerides due to impaired lipogenesis [13]. In murine brown adipocytes in primary culture, we found that EFV also impairs adipocyte differentiation, an effect that was associated with down-regulation of marker genes for adipogenesis, including fatty acid binding protein-4 (FABP-4) and peroxisome proliferator-activated receptor-γ (PPARγ) [14]. In addition to these data in mouse adipocytes, El Hadri *et al*. [13] reported morphological changes indicative of impaired adipogenesis in human adipocytes in primary culture, but a parallel comparative study of the effects of HAART therapy components, EFV and LPV/r, in human adipocytes is lacking.

A major mechanism by which alterations in adipose tissue result in metabolic systemic alterations is the release of regulatory molecules by fat depots. At present, adipose tissue is recognized as an endocrine organ because it releases multiple regulatory proteins that affect not only the surrounding tissue but also target organs such as liver, muscle and brain. Pre-adipocytes and adipocytes within adipose stores act as sources of adipokines (proteins released preferentially by adipose cells that have long-distance actions on other organs) as well as inflammation-related cytokines (which act both locally and at a distance) [15, 16]. Local and systemic alterations mediated by adipose tissue in response to pathogenic insults, such as drugs or viral infection, may occur through altered release of pro-inflammatory cytokines and adipokines [1]. Some reports indicate that ritonavir, in addition to impairing adipocyte differentiation and gene expression, impairs the secretion of adiponectin and increases the release of the inflammatory cytokines MCP-1 and interleukin-6 [9, 17]. However, the effects of EFV have not been determined. In the present study, we undertook a comparative analysis of the effects of EFV and LPV/r on human adipocytes in culture, evaluating adipogenic differentiation, gene expression and release of selected cytokines and adipokines.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagles (DMEM)/F12 medium was from Gibco. All reagents used for adipocyte cell cultures were from Sigma, with the exception of rosiglitazone, which was from Cayman Chemicals. EFV was supplied by Bristol-Myers-Squibb, and lopinavir and ritonavir were from Abbott. Drugs were dissolved in dimethylsulfoxide (DMSO). The concentration of drug added to the medium essentially corresponds to the concentration available to cells, since differentiating cell culture medium is devoid of added serum or albumin capable of binding drugs, and protein concentration in the medium was practically undetectable (see below). For controls, equal amounts of DMSO were added to cell cultures.

Cell Culture and Treatment. Human pre-adipocytes were obtained from Advancell (Barcelona, Spain). Preadipocyte preparations corresponded to pooled cells from 2-5 different young (around 30 years old) healthy female donors. Cells were cultured and differentiated as previously reported [18], with slight modifications. Briefly, pre-adipocytes at 80% confluence were treated with differentiation culture medium (DMEM/F12, 33 μM biotin, 17 μM sodium panthotenate, 200 nM insulin, 25 nM dexamethasone, 0.5 mM IBMX, 2 μM rosiglitazone, 0.2 nM triiodothyronine) to induce differentiation (day 0 of differentiation). After 4 days, and every 5 days thereafter, the medium was replaced with fresh culture medium with the same composition but without IBMX, rosiglitazone and dexamethasone. Under these conditions, maximal differentiation, defined as the maximal amount of cell culture surface occupied by cells containing lipid droplets, was attained in controls 15 days after induction (see Fig. **1**). Experiments were performed using two experimental settings. For studies on the effects of drugs on adipocyte differentiation, treatment was initiated on day 0 and continued during the entire differentiation process. Fresh drugs were included every time medium was replaced. The extent of morphological differentiation under distinct experimental conditions was quantified by measuring the percentage of cell culture surface occupancy by adipocytes in relation to controls, which were set at 100%. For studies on the effects of drugs on differentiated adipocytes, drugs were added on day 15 after initiation of adipogenic differentiation (maximal differentiation stage), and cells were studied after treating for 24 hours. The range of concentrations of drugs tested were chosen on the basis of cytotoxicity data in the human pre-adipocyte and adipocyte cell cultures (see Results), and available data on the range of EFV and LPV/r concentrations in plasma and adipose tissue from tretated patients [19, 20, 21].

Quantitative Real-Time RT-PCR. RNA was extracted using an RNeasy Mini Kit (Qiagen). Reverse transcription was performed in a total volume of 20 μl using random hexamer primers (Applied Biosystems) and 0.5 μg total RNA. PCR reactions contained 1 μl cDNA, 12.5 μl TaqMan Universal PCR Master Mix, 250 nM probes and 900 nM primers from Assays-on-Demand Gene Expression Assay Mix (TaqMan, Applied Biosystems) in a total volume of 25 μl, and were conducted using an ABI/Prism 7700 Sequence Detector System. The Assay-on-Demand probes used were: cytochrome c oxidase subunit IV (COX4I1), Hs00266371; CEBPα, Hs00269972; lipoprotein lipase, Hs00173425; TNFα, Hs00174128; PPARγ, Hs00234592; adiponectin, Hs00605917; Pref-1, Hs00171584; leptin, Hs00174877; MCP-1, Hs00234140, and 18S rRNA, Hs99999901. Cytochrome c oxidase subunit II (COII) mRNA levels were quantified using the primers: 5'-CAA ACC ACT TTC ACC GCT ACA C-3' (forward) and 5'-GGA CGA TGG GCA TGA AAC TGT-3' (reverse), and the FAM-labeled probe, 5'-AAA TCT GTG GAG CAA ACC-3', designed using the Assay-by-design system (Custom TaqMan Gene Expression Assays, Applied Biosystems, USA). Mitochondrial DNA (mtDNA), quantified using these primers/probes, was expressed relative to nuclear DNA levels, determined by amplification of the intronless gene, C/EBPα. Controls lacking RNA, primers, or reverse transcriptase were included in each set of experiments. Each sample was run in duplicate and the mean value of the duplicate was used to calculate the

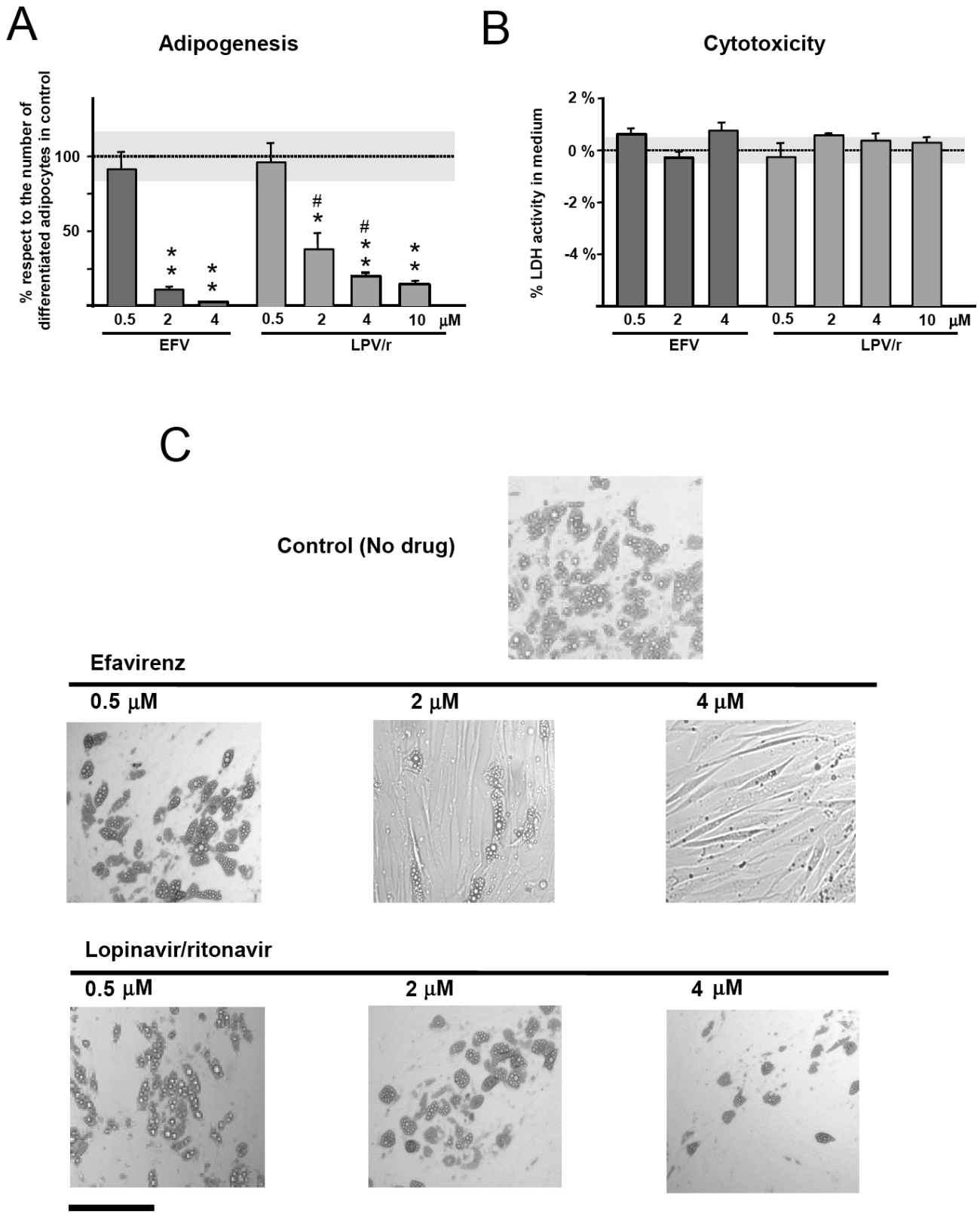

Fig. (1). (**A**) Quantitative assessment of the effects of Efavirenz or Lopinavir/ritonavir (4:1) (LPV/r) treatment on adipogenic differentiation. Quantification was performed through image analysis of percent of surface occupancy by adipocytes using 5-7 random micrographs from independent cultures referred to control values that were set to 100. (**B**) Cytotoxicity, expressed as percentage of LDH released to the medium respect to maximal release in totally lysed cells (see Methods section). In A and B panels, bars are means ± SEM, and controls are shown as dotted line, mean; shaded area, ± SEM. Statistical significance of differences with respect to controls are denoted by ** ($P < 0.01$). Significant differences between EFV at LPV/r at the same concentration are denoted by $^{\#}(P < 0.05)$. (**C**) Representative micrographs of human pre-adipocytes differentiated in culture in the presence of the indicated concentrations of drugs. The scale bar corresponds to 200 μm.

relative amount of individual target. Each mean value was normalized to that of the 18S rRNA gene using the comparative ($2^{-\Delta CT}$) method, following the manufacturer's instructions.

Quantification of Adipokines, Cytokines and Lactate in Adipocyte Culture Medium. For the experimental setting involving drug treatment during the adipocyte differentiation process, release of adiponectin, leptin, monocyte chemoattractant protein-1 (MCP-1), interleukin-6, interleukin-8, total plasminogen activator inhibitor type-1 (PAI-1), hepatocyte growth factor (HGF) and nerve growth factor (NGF) was determined using 25 µl of medium collected during the last 5 days of culture before harvest. The levels of these factors in the medium were quantified using a multiplex analysis system based on fluorescently labeled microsphere beads linked to specific antibodies (Linco Research/Millipore); fluorescence was detected using Luminex100ISv2 equipment. The levels of interleukin-1b, resistin and TNF-α, which could also be determined by the multiplex system used (HADCYT-61K, Millipore), were below the detection limits in the adipocyte culture medium under all conditions tested. Lactate in the medium was measured spectrophotometrically (Roche). Sample analyses were performed in duplicate.

Cytotoxicity Assays. Potential cytotoxic effects of drugs on differentiating human pre-adipocytes or on adipocytes when already differentiated, were determined by the CytoTox96 kit (Promega) following manufacturer instructions and after exposure of differentiating (5 days) or already differentiated adipocytes (24h) to the drugs. Basically, the assay quantitatively measures lactate dehydrogenase (LDH) in the cell culture medium. LDH is a stable cytosolic enzyme that is released upon cell lysis, and which utility for measuring cytotoxicity has been validated previously [22, 23]. Data were calculated as percentage of LDH activity in the medium after exposure to drugs respect to the maximum release after total lysis of cells, in accordance with manufacturer instructions.

Statistical Analysis. Where appropriate, Student's *t*-tests were used to determine significance between groups; significance is indicated in the text.

RESULTS

Effects of EFV and LPV/r on Morphological Differentiation of Human Adipocytes. A quantitative assessment of the extent of differentiation of drug-treated human adipocytes relative to non-treated controls (defined as 100%) is shown in Fig. (**1A**). Microscopy images representative of treatment-induced changes in the acquisition of adipocyte morphology during the *in vitro* differentiation process are shown in Fig. (**1C**).

EFV did not alter the extent of morphological adipocyte differentiation at 0.5 µM, but severely impaired morphological adipocyte differentiation at 2 and 4 µM. These effects occurred without significant cytotoxic effects (Fig. **1B**). Exposure of cells to 10 µM EFV caused extensive cell death, thus precluding further analysis of differentiation effects at higher EFV concentrations. LPV/r did not affect morphological differentiation at 0.5 µM, but induced a progressive dose-dependent impairment of differentiation at higher concentrations; at 10 µM, the highest dose tested, only very low levels of differentiation were observed (Fig **1A**). However, there were no significant cytotoxic effects of LPV/r at any of the concentrations tested (Fig. **1B**). Thus, at this level of analysis, the major distinctions between EFV and LPV/r effects were: a) EFV was toxic for adipogenic cells at 10 µM, whereas LPV/r was not; and b) at equivalent concentrations of EFV and LPV/r (2 and 4 µM), EFV always caused a greater impairment of morphological adipogenic differentiation than did LPV/r.

Effects of EFV and LPV/r on Gene Expression in *In Vitro*-Differentiating Human Adipocytes. Consistent with the observed morphological effects, treatment of human adipocytes with EFV during differentiation caused a progressive impairment in the expression of the mRNAs for genetic markers of adipogenic differentiation, including adiponectin, leptin and lipoprotein lipase (Fig. **2**). Similarly, the mRNA levels of the adipogenesis master regulator genes PPARγ and CCAAT/Enhancer binding protein-α (C/EBPα) were also reduced. These effects were observed at concentrations as low as 0.5 µM, which induced a significant reduction in PPARγ and lipoprotein lipase mRNA levels. At 2 µM and 4 µM, EFV induced a marked reduction in the levels of adiponectin, PPARγ, lipoprotein lipase, C/EBPα, and leptin mRNAs. In fact, in cells treated with 4 µM EFV, expression of most of these marker genes was less than 20% of control values (Fig. **2**).

Treatment of human adipocytes with 4 or 10 µM LPV/r during differentiation also caused a moderate, but significant, reduction in the expression of adiponectin, leptin and PPARγ; at lower concentrations (0.5 and 2 µM) LPV/r had no effect. C/EBPα mRNA expression levels were only significantly reduced with 4 µM LPV/r. In general, the reduction in expression of these adipogenesis marker genes was 50–75% that of control values (Fig. **2**). For lipoprotein lipase mRNA, the reduction was greatest (~30%) and most significant at lower concentrations (2 µM) of LPV/r.

The mRNA for the anti-adipogenic molecular marker Pref-1 was not detectable in adipocytes under any condition tested, even in completely non-differentiated cells (e.g., at a high concentration of EFV).

The mRNA levels of the mitochondrial toxicity markers COII and COIV, and the levels of mtDNA were unaltered at all EFV and LPV/r concentrations tested. The mRNA levels of the pro-inflammatory cytokine MCP1 were induced relative to controls at the concentrations of EFV and of LPV/r tested. However, EFV induced a greater increase in MCP1 mRNA (> 10-fold induction at 2 and 4 µM) than did LPV/r (2–3-fold induction) (Fig. **2**). TNFα mRNA was undetectable under all conditions tested.

In summary, at a given concentration of drug, EFV either repressed adipogenic gene expression more profoundly than did LPV/r (2 and 4 µM for adiponectin, PPARγ and lipoprotein lipase), or was the only one of the two drugs that showed repressive effects (0.5 µM for PPARγ and lipoprotein lipase). A similar result was obtained for the pro-inflammatory marker MCP-1, the expression of which was consistently induced to a greater degree by EFV than LPV/r at each of the concentrations tested.

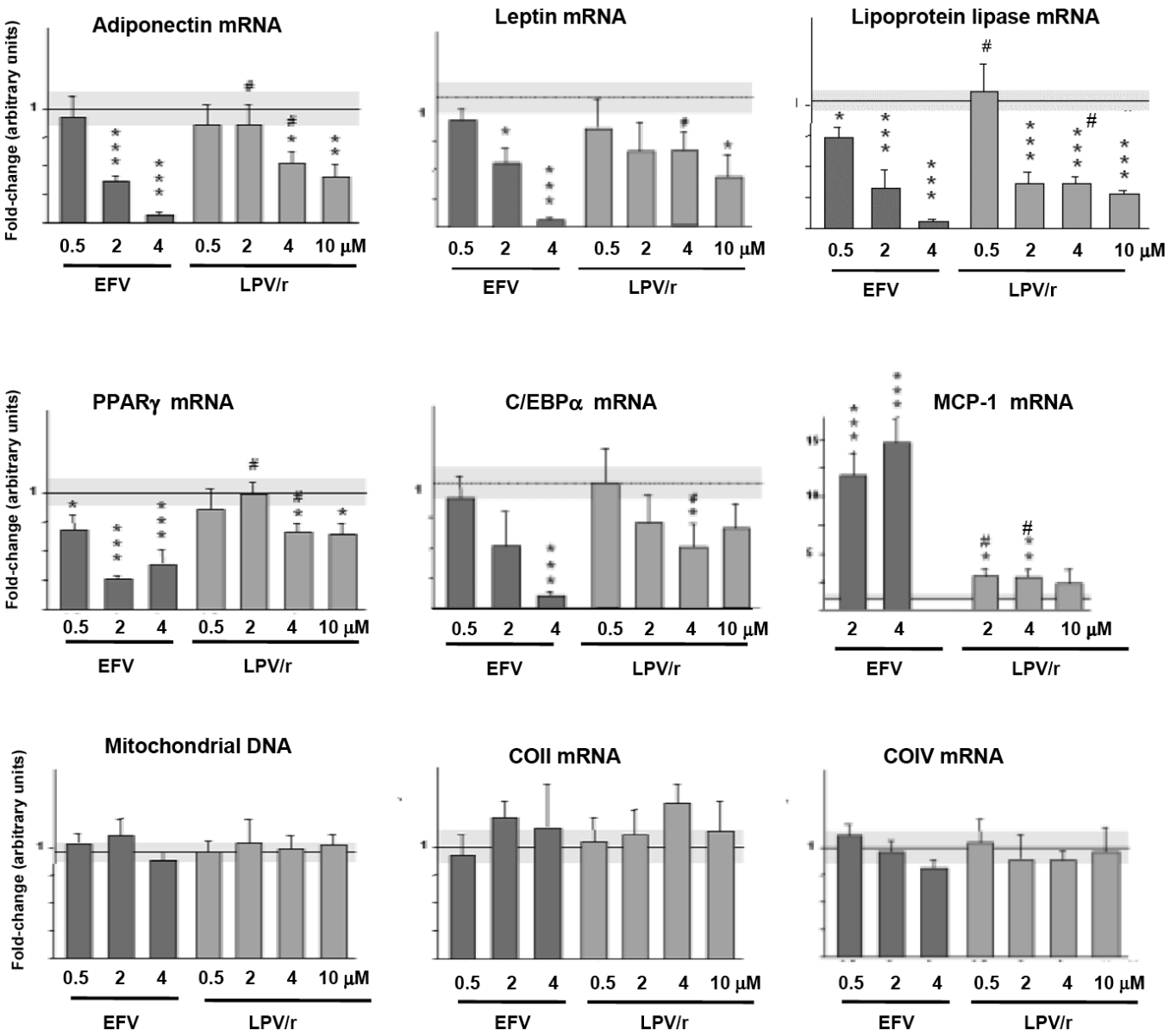

Fig. (2). Effects of EFV and LPV/r on mtDNA levels, and the expression of genes related to mitochondrial function and adipogenic function in human adipocytes differentiating in culture. Human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs. Data are shown as means ± SEM from 4 to 5 independent experiments, and are expressed relative to values from untreated control cells, which were set to 1 (dotted line, mean; shaded area, ± SEM). Statistical significance of differences with respect to controls are denoted by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$). Significant differences between EFV at LPV/r at the same concentration are denoted by are denoted by $^{\#}(P < 0.05)$.

Effects of EFV and LPV/r on the Secretion of Adipokines, Cytokines and Lactate by Differentiating Human Adipocytes. The effects of equivalent concentrations of EFV and LPV/r (4 μM) on the release of regulatory proteins and lactate were studied (Fig. **3**). EFV caused a marked increase in the levels of MCP-1 (5.3-fold), interleukin-6 (4.2-fold), interleukin-8 (18.7-fold), PAI-1 (5.7-fold) and HGF (5.5-fold) in the culture medium compared with untreated adipocytes. NGF levels were unaltered. In contrast, EFV treatment markedly decreased the release of adiponectin and leptin, reducing protein levels in the medium to 17% and 19% that of control values, respectively. LPV/r caused small but significant reductions in the release of adiponectin (29% reduction) and leptin (24% reduction). Adiponectin and leptin levels were significantly greater in the medium from LPV/r-treated adipocytes than in medium from EFV-treated cells. Unlike EFV, LPV/r did not increase significantly the release of MCP-1, interleukin-8 or PAI-I. Both HGF and interleukin-6 were induced to a similar extent by each treatment, and neither drug had any effect on lactate release (Fig. **3**).

Effects of EFV and LPV/r on Previously Differentiated Human Adipocytes. In addition to studying the effects of EFV and LPV/r during the adipocyte differentiation process, we also analyzed the action of the drugs on human adipocytes that had already differentiated. LPV/r did not modify the morphology of differentiated adipocytes at any of the concentrations tested as well as it

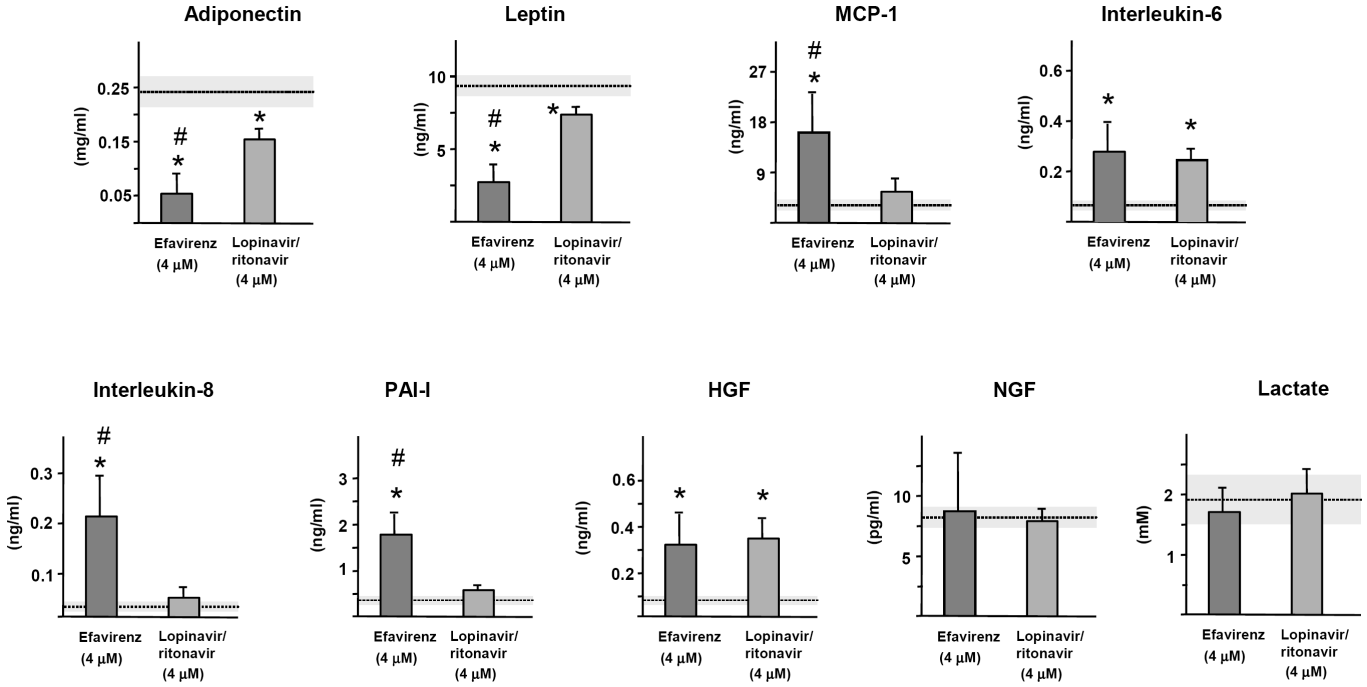

Fig. (3). Effects of EFV and LPV/r on the release of selected cytokines, adipokines and lactate by human adipocytes differentiating in culture. Human pre-adipocytes were differentiated in culture in the presence of the indicated concentrations of drugs. Data are shown as means ± SEM from 4 to 5 independent experiments, and are expressed relative to values from untreated control cells, which were set to 1 (dotted line, mean; shaded area, ± SEM). Statistical significance of differences with respect to controls are denoted by * ($P < 0.05$) Significant differences between EFV at LPV/r at the same concentration are denoted by are denoted by $^{\#}(P < 0.05)$.

did not cause significant cytotoxic effects (Fig. **4A**). Adipocyte morphology was also not modified by EFV at any of the concentrations analyzed except 20 μM, which resulted in marked cell death (cytotoxicity close to 80%, data not shown). EFV at 10 μM and at lower concentration (4 μM) did not cause significant cytotoxic effects (Fig. **4B**). The effects of 4 and 10 μM EFV and LPV/r on gene expression in differentiated adipocytes were also determined (Fig **4C**). EFV caused a marked repression of adiponectin and PPARγ gene expression, whereas LPV/r only modestly reduced PPARγ mRNA levels and did not alter adiponectin gene expression. Thus, at equivalent concentrations, EFV decreased adiponectin and PPARγ levels to significantly lower levels in differentiated adipocytes than did LPV/r. The expression levels of both mtDNA-encoded (COII mRNA) and nuclear DNA-encoded (COIV mRNA) (marker genes of mitochondrial function) were virtually unchanged by drug treatment, although EFV induced a very modest reduction at 2 μM. Both drugs increased MCP-1 mRNA levels relative to controls; although the induction by EFV tended to be greater than did that by LPV/r, this difference was not significant.

DISCUSSION

The present findings show that both EFV and LPV/r impair *in vitro* differentiation of human adipocytes, but clearly demonstrate that this effect is greater with EFV. The negative effect of EFV and LPV/r on human adipocyte differentiation was shown by impaired acquisition of adipocyte morphology and overall reductions in the expression levels of adipogenesis marker genes, and this occurred at drug concentrations that did not cause cytotoxic effects. This is exemplified by reduced expression of the gene for lipoprotein lipase, a key enzyme for lipid accretion in adipocytes, and a reduction in adiponectin and leptin mRNA levels; adiponectin and leptin proteins are characteristic of differentiated adipocytes. The most likely explanation for these overall effects of EFV and LPV/r on adipogenesis can be found in the observation that these drugs also down-regulated the adipogenesis master genes C/EBPα and PPARγ, particularly the latter. These transcription factors are known to exert concerted effects during adipogenesis, promoting the acquisition of adipocyte morphology and metabolic characteristics [15, 24]. Consistent with this interpretation, the preferential reduction of C/EBPα and PPARγ mRNA by EFV compared with LPV/r paralleled the more profound overall effects of EFV on adipogenesis. An expansion of the study of EFV effects to the analysis of the action of these drugs on previously differentiated adipocytes showed that EFV caused a greater reduction in the expression levels of PPARγ and its target adiponectin.

The reduced expression of adiponectin and leptin in response to EFV, and to a lesser extent in response to LPV/r, raises the possibility of systemic effects as a consequence of drug treatments, considering that these adipokines are released to the circulation by adipocytes. Our present data on the release of adiponectin and leptin proteins to the medium confirmed the results of the gene expression analysis: both EFV and LPV/r reduced the release of these adipokines by adipocytes and again, the effects of EFV were more marked. This is especially relevant for adiponectin because lower levels of serum adiponectin are commonly observed in HIV-1-infected, HAART-treated patients with overt or milder lipodystrophy symptoms [25]. Because adiponectin is an

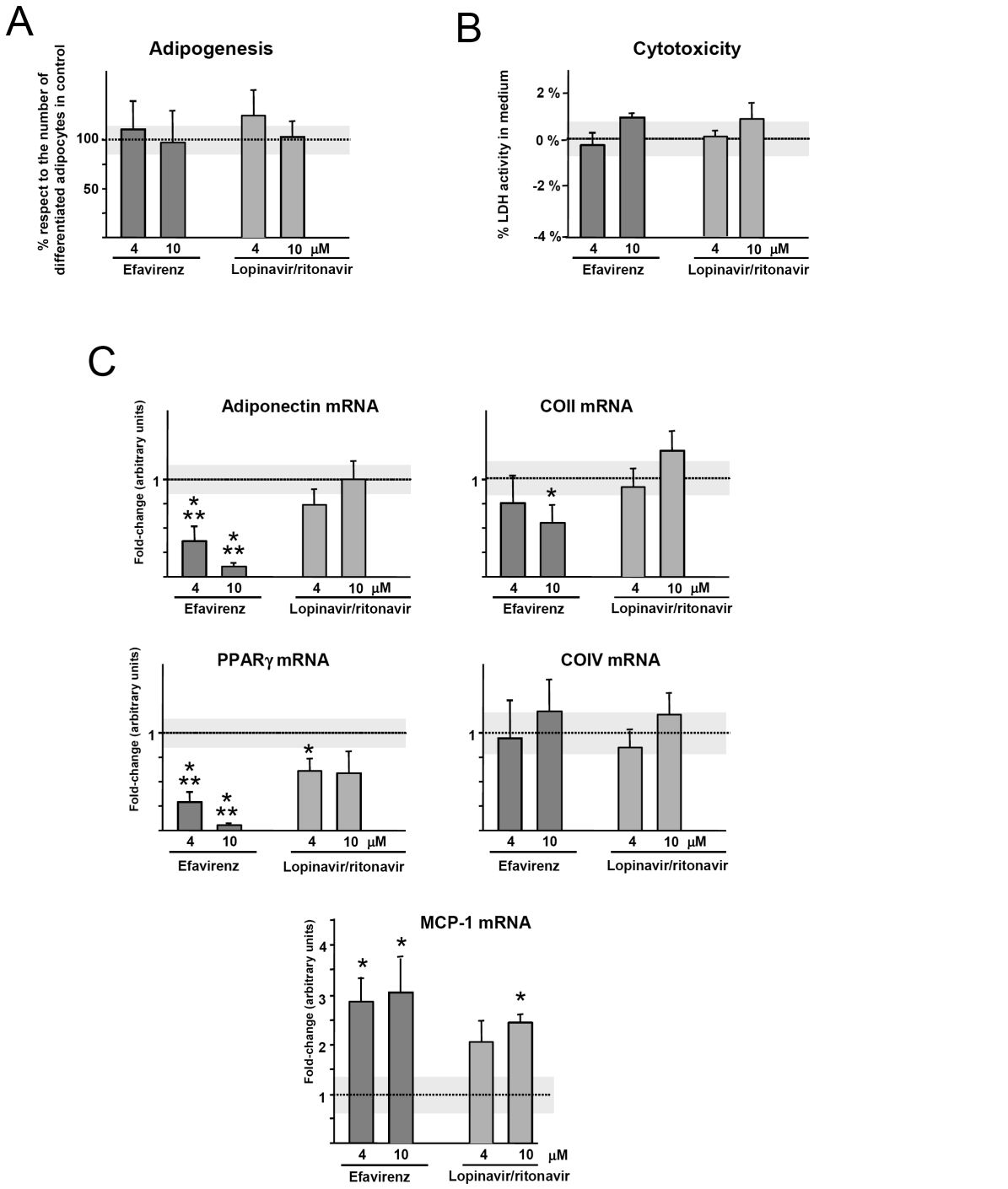

Fig. (4). A, Quantitative assessment of the effects of Efavirenz or Lopinavir/ritonavir (4:1) (LPV/r) treatment on differentiated adipocytes. Quantification was performed through image analysis of percent of surface occupancy by adipocytes using 5-7 random micrographs from independent cultures referred to control values that were set to 100. B, Cytotoxicity, expressed as percentage of LDH released to the medium respect to maximal release in totally lysed cells (see Methods section). C, Effects of EFV and LPV/r on the expression of genes related to mitochondrial function and adipogenic function in differentiated human adipocytes. Data are shown as means ± SEM from 4 to 5 independent experiments, and are expressed relative to values from untreated control cells (dotted line, mean; shaded area, ± SEM). Statistical significance of differences with respect to controls are denoted by * ($P < 0.05$) and *** ($P < 0.001$). Significant differences between EFV at LPV/r at the same concentration are denoted by $^{\#}(P < 0.05)$.

insulin-sensitizing agent [26], EFV-induced repression of adiponectin expression and release must be viewed in relation to the potential diabetogenic effects of antiretroviral treatments. The fact that adiponectin is known to possess powerful anti-inflammatory effects [27] also suggests that a reduction in adiponectin levels, caused preferentially by EFV, could contribute to a more pro-inflammatory environment. This is in addition to the direct stimulatory effect of EFV on pro-inflammatory adipokine expression and release.

Both EFV and LPV/r caused important effects on the expression and release of pro-inflammatory cytokines. Interleukin-6 and MCP-1, pro-inflammatory cytokines released by pre-adipocytes and adipocytes [28, 29], are considered to be important determinants of the pro-inflammatory status of adipose tissue in HIV-1/HAART-associated lipodystrophy [2, 26]. Levels of MCP-1 mRNA expression and release of the MCP-1 protein by adipocytes were enhanced as a consequence of both treatments, but the effects were greater with EFV. Similar effects were observed for interleukin-8, a pro-inflammatory cytokine produced by pre-adipocytes and adipocytes (in addition to macrophages) and released by adipose tissue. Significantly, the levels of local and systemic interleukin-8 are increased in HIV-1/HAART-associated-lipodystrophy [30]. PAI-1, a natural inhibitor of fibrinolysis that is produced by adipose tissue and activated in serum after proteolysis, is increased in plasma from patients with HIV-1/HAART-associated lipodystrophy [31]. Here again, the present data indicate that EFV induced a greater increase in PAI-1 release by human adipocytes than did LPV/r. Collectively, these findings indicate that, although both EFV and LPV/r induce the expression and release of pro-inflammatory cytokines, the effect is always greater with EFV exposure, and is sometimes only seen with EFV. It should be noted that the effects of antiretroviral drugs and the more intense action of EFV were not uniform across all regulatory molecules released by adipocytes. For instance, the release of NGF, a neurotrophin also produced by adipose tissue and induced in association with obesity and the metabolic syndrome [32], was unaltered in response to either of the two antiretroviral treatments tested. In contrast, HGF release was equally induced by EFV and LPV/r. HGF is a powerful angiogenic factor that is produced by adipose tissue and the expression of which is increased in the obesity-induced inflammation of adipose tissue [33]. However, no data about HGF in HIV-1-infected patients under HAART are available. Further research will be needed to assess the potential disturbances in HGF levels in HIV-1-infected patients in light of the present findings.

As expected, all the effects of EFV and LPV/r occurred in the absence of mitochondrial toxicity. mtDNA levels, and the expression of mtDNA-encoded and nuclear DNA-encoded transcripts, were essentially unaffected by EFV or LPV/r. The release of lactate by adipogenic cell cultures, an indicator of the extent to which mitochondrial oxidative metabolism is impaired, was also unaffected by EFV or LPV/r, whereas exposure of human adipogenic cells to 20 μM didanosine, a positive control for nucleoside-analog reverse transcriptase inhibitor-induced mitochondrial impairment [14], resulted in a 3.8-fold increase in lactate release to the medium (data not shown).

In summary, the present data indicate that EFV powerfully impairs human adipocyte differentiation, decreasing the release of anti-diabetic adipokines, such as adiponectin, increasing the release of pro-inflammatory cytokines and decreasing the expression and release of anti-diabetic and anti-inflammatory adipokines, such as adiponectin. At equivalent concentrations, the effects of LPV/r were markedly less than those of EFV. Notably, the effects of EFV on differentiating adipocytes described here occurred at concentrations ranging from 0.5 to 4 μM.

The present study has obvious limitations in relation to the translation of the present *in vitro* data to patient treatment considerations. The average EFV plasma concentrations is approximately 10 μM in patients and the minimum efficacious concentration has been established as 3 μM [19], indicating that *in vitro* findings may have *in vivo* relevance. However, the EFV is added to adipocyte cell cultures in a serum-free medium (a culture condition intrinsically required for differentiation *in vitro* of human adipocytes), and this may result in a higher availability of EFV to cells due to lack of binding to proteins. However, EFV has been reported to accumulate in adipose tissue from patients at a range close to 100 nmol/g tissue [20]; thus, intracellular concentrations may be higher than plasma concentrations [20, 21]. On the other hand, LPV/r can hardly achieve intracellular concentrations higher than the 1μM range inside adipose tissue [20]. In any case, the present results are consistent with recent data indicating that lipoatrophy is more common with EFV than LPV/r when these drugs are combined with thymidine-analog reverse transcriptase inhibitors [3], and may have implications for the pathogenesis of HIV-1-associated lipodystrophy and for the improvement of anti-retroviral treatments to minimize adipose tissue and metabolic alterations in HIV-1-infected patients.

ACKNOWLEDGEMENTS

This work was supported by Fondo de Investigaciones Sanitarias (PI081715) and Red de Investigación en SIDA (RD06/006/0022), Instituto de Salud Carlos III, FIPSE (36610/06), Spain, and an independent grant from Abbott Laboratories. This pharmaceutical firm had no role in the study design, data collection and interpretation or writing of the article..

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Received: May 21, 2010 **Revised: October 13, 2010** **Accepted: October 25, 2010**

Effects of nevirapine and efavirenz on human adipocyte differentiation, gene expression, and release of adipokines and cytokines

Revista: Antiviral Research. 2011 Aug; 91(2):112-9. Epub 2011 May 17.

Título: Efectos de la nevirapina y el efavirenz en la diferenciación de adipocitos humanos, expresión génica y secreción de adipoquinas y citoquinas.

PMID: 21619898

Los inhibidores de transcriptasa reversa no análogos de nucleósido (NNRTI) nevirapina y efavirenz son fármacos utilizados en la terapia antirretroviral para la infección por el virus HIV-1. A pesar de que los NNRTI no han sido tradicionalmente asociados con la aparición de alteraciones en el tejido adiposo, datos recientes sugieren que el efavirenz podría contribuir a las alteraciones del tejido adiposo de pacientes bajo tratamiento antirretroviral, de acuerdo con su capacidad para inhibir la diferenciación de adipocitos en cultivo. Estos efectos no se han descrito para la nevirapina, el otro NNRTI más comúnmente usado. En este estudio determinamos los efectos de la nevirapina en la diferenciación, expresión génica y secreción de proteínas reguladoras (adipoquinas y citoquinas) de adipocitos humanos en diferenciación y los comparamos con los efectos del efavirenz. El efavirenz provoca una represión dosis-dependiente de la diferenciación adipocitaria que está asociada a la represión de genes reguladores de la adipogénesis como SREBP-1, PPARγ y C/EBPα y sus dianas como son la lipoproteina lipasa, la leptina y la adipoquina que son proteínas clave en el funcionamiento de los adipocitos. En contraste, la nevirapina no afecta a la adipogénesis y genera un aumento moderado pero significativo en la expresión de SREBP-1, PPARγ y C/EBPα y sus genes diana solo a una concentración de 20μM. Además el efavirenz provoca un aumento significativo en la secreción de citoquinas pro-inflamatorias (IL8, IL6 y MCP1), PAI-1 y HGF, mientras la nevirapina no provoca cambios en la secreción de estos factores, menos IL6 y HGF cuya secreción se ve disminuida con la nevirapina. La nevirapina induce la secreción de adipoquina mientras el efavirenz la reprime severamente. Además la nevirapina inhibe la actividad trasncriptasa reversa endógena de los preadipocitos mientras el efavirenz no altera esta actividad. Se concluye que en contraste con los efectos anti-adipogénicos y pro-inflamatorios del efavirenz, la nevirapina no reprime la adipogenesis.

Antiviral Research 91 (2011) 112–119

Contents lists available at ScienceDirect

Antiviral Research

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Effects of nevirapine and efavirenz on human adipocyte differentiation, gene expression, and release of adipokines and cytokines

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ARTICLE INFO

Article history:
Received 23 July 2010
Revised 10 February 2011
Accepted 19 April 2011
Available online 17 May 2011

Keywords:
Lipodystrophy
Adipocyte
Nevirapine
Efavirenz
Non-nucleoside analog reverse transcriptase inhibitor
Adipokine

ABSTRACT

The non-nucleoside reverse transcriptase inhibitors (NNRTIs) nevirapine and efavirenz are drugs of choice for initial antiretroviral treatment for HIV-1 infection. Although NNRTIs have not traditionally been associated with the appearance of adipose alterations, recent data suggest that efavirenz may contribute to adipose tissue alterations in antiretroviral-treated patients, consistent with its ability to impair differentiation of adipocytes in cell cultures. No such effects have been reported for nevirapine, the other most commonly used NNRTI. In this study, we determined the effects of nevirapine on differentiation, gene expression and release of regulatory proteins (adipokines and cytokines) in differentiating human adipocytes, and compared them with those of efavirenz. Efavirenz caused a dose-dependent repression of adipocyte differentiation that was associated with down-regulation of the master adipogenesis regulator genes SREBP-1, PPARγ and C/EBPα, and their target genes encoding lipoprotein lipase, leptin and adiponectin, which are key proteins in adipocyte function. In contrast, nevirapine does not affect adipogenesis and causes a modest but significant coordinate increase in the expression of SREBP-1, PPARγ and C/EBPα and their target genes only at a concentration of 20 μM. Whereas efavirenz caused a significant increase in the release of pro-inflammatory cytokines (interleukin [IL]-8, IL-6, monocyte chemoattractant protein-1), plasminogen activator inhibitor type-1 and hepatocyte growth factor (HGF), nevirapine either had no effect on these factors or decreased their release (IL-6 and HGF). Nevirapine significantly increased adiponectin release, whereas efavirenz strongly repressed it. Moreover, nevirapine inhibited preadipocyte endogenous reverse transcriptase activity, whereas efavirenz did not alter it. It is concluded that, in contrast with the profound anti-adipogenic and pro-inflammatory response elicited by efavirenz, nevirapine does not impair adipogenesis.

1. Introduction

Alterations in adipose tissue distribution (lipodystrophy syndrome) and systemic metabolic disturbances (dyslipidemia and insulin resistance) appear frequently in HIV-1-infected patients under highly active antiretroviral treatment (HAART). HAART employs a drug regimen that typically includes nucleoside-analog reverse transcriptase inhibitors (NRTIs), protease inhibitors (PIs) and/or non-nucleoside analog inhibitors of reverse transcriptase (NNRTIs). The complex metabolic alterations that arise with these treatment regimens reflect the action of HAART drug combinations on the susceptible status of HIV-1-infected patients. Although individual drug treatments cannot account for the development of the lipodystrophy syndrome, NRTIs, such as stavudine and zidovudine, are thought to predispose toward peripheral lipoatrophy, whereas PIs are considered to favor insulin resistance and dyslipidemia (Villarroya et al., 2005). NNRTIs are drugs of choice for initial antiretroviral treatment of HIV-1-infected patients, in combination with drugs from other families. Unlike NRTIs, NNRTIs do not inhibit DNA polymerase-γ, and therefore are not expected to elicit mitochondrial toxicity, a major suspected cause of adipose tissue alterations in HAART-treated HIV-1-infected patients.

Efavirenz, an NNRTI, is the preferred third agent to include in antiretroviral regimes according to most international antiretroviral treatment guidelines (Hammer et al., 2008; Gazzard et al., 2008). This is because efavirenz has never been surpassed in clinical trials, and, in fact, has shown better antiretroviral efficacy than PIs in pivotal clinical trials (Staszewski et al., 1999; Riddler et al., 2008). Nevirapine, though more restricted in use than efavirenz, is the other NNRTI commonly used in several European countries, especially in developing countries where a compact pill with stavudine and lamivudine is often used for initial antiretroviral therapy (Zhou et al., 2007; Colebunders et al., 2005). The 2NN

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doi:10.1016/j.antiviral.2011.04.018

study was originally intended to directly compare efavirenz and nevirapine (van Leth et al., 2004a); unfortunately, however, no conclusion could be drawn from this study in terms of non inferiority between both agents.

NNRTIs have not traditionally been associated with the appearance of lipodystrophy and are often perceived as benign in terms of adipose alterations. However, recent clinical trials have shown that efavirenz could favor lipoatrophy when used as a component of the HAART cocktail (Riddler et al., 2008; Haubrich et al., 2009). In contrast, several reports have indicated that shifting PI- or NRTI-based regimes to one containing nevirapine modestly ameliorated disturbances in the lipid profile of adults (Negredo et al., 1999) or pediatric (Gonzalez-Tome et al., 2008) patients. Switching to nevirapine also caused minor improvements in lipodystrophy (Negredo et al., 1999), but did not attenuate specific events associated with lipoatrophy, such as adipose tissue apoptosis (Domingo et al., 2001). Nevirapine appears to cause a better improvement in the lipid profile of treated patients respect to efavirenz (Fisac et al., 2005; van Leth et al., 2004b), basically by increasing HDL-cholesterol and the total cholesterol/HDL-cholesterol ratio.

There are few studies on the effects of NNRTIs on adipocytes. Using murine cell models, we reported (Rodríguez de la Concepción et al., 2005) that nevirapine favors differentiation and expression of marker genes of adipocyte function, such as peroxisome proliferator-activated receptor gamma (PPARγ), in primary brown adipocytes. This was in contrast to the anti-adipogenic effect of efavirenz in these cells. Efavirenz has been reported to decrease the capacity of mouse 3T3-L1 preadipocytes to accumulate triglycerides due to impaired lipogenesis (El Hadri et al., 2004), whereas no effects were observed for nevirapine in the murine adipogenic cell line 3T3-F422A (Caron et al., 2004). Similarly, studies of differentiating human adipocytes, which have been limited to reports on morphological changes, have shown reduced adipogenesis with efavirenz treatment (El Hadri et al., 2004) and no adipogenic effects of a single concentration (10 μg/ml) of nevirapine (Vernochet et al., 2005).

One of the main physiopathogenic components of the interplay between adipose tissue disturbances and systemic metabolic derangements in response to viral and pharmacological insults is interference with the secretory functions of adipose tissue. Adipose tissue is not only a site of fat storage, but is also responsible for releasing regulatory factors such as adipokines and pro-inflammatory cytokines that act both locally and on distant organs (e.g., liver, muscle, heart, pancreas) to influence overall metabolism. For instance, adipose tissue releases the insulin-sensitizing hormone adiponectin and other factors that affect insulin resistance, such as resistin, as well as inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin (IL)-6 (Hauner, 2005). Thus, the potential action of drugs on adipose tissue may not only affect adipose tissue development, it can also alter adipose tissue secretory functions, and thereby alter systemic metabolism.

The effects of the NNRTIs nevirapine and efavirenz on the secretory functions of adipocytes have not been investigated to date. In the present study, we conducted a comparative analysis of nevirapine and efavirenz action on adipocyte differentiation, gene expression and release of adipokines and cytokines by human adipocyte cells in culture.

2. Methods

2.1. Preadipocyte differentiation and culture

Human adipocyte precursor cells from healthy individuals, obtained from Advancell (Barcelona, Spain), were cultured as previously reported (Schlüter et al., 2002). Differentiation was induced by treating cells at 80% confluence with Dulbecco's modified Eagle's (DMEM)/F12 medium containing 33 μM biotin, 17 μM sodium pantothenate, 200 nM insulin, 25 nM dexamethasone, 0.5 mM IBMX (3-isobutyl-1-methylxanthine), 2 μM rosiglitazone, and 0.2 nM triiodothyronine. After 4 days, the medium was replaced with culture medium with the same composition but without IBMX, rosiglitazone or dexamethasone; thereafter, medium was replaced every 5 days. In untreated cells, maximal differentiation, estimated from the maximal percentage of cells showing lipid droplet accumulation, was attained 15 days after induction of differentiation. Treatment with nevirapine or efavirenz was initiated on day 0 and was maintained throughout the differentiation process. Fresh drugs (dissolved in DMSO) were included with each change of medium. An equal amount of DMSO (<0.1%) was added to all control cell cultures.

2.2. Assessment of cytotoxicity

Potential cytotoxic effects of drugs on differentiating human preadipocytes were determined using a CytoTox96 kit (Promega, Madison, WI) following the Manufacturer's instructions.

2.3. Assessment of preadipocyte differentiation

The extent of morphological differentiation was quantified by measuring the percentage of the cell culture surface occupied by adipocytes relative to controls (defined as 100%). Adipocyte differentiation was also quantified after 15 days by measuring the intracellular lipid accumulation after Oil Red O staining, as reported elsewhere (Laughton, 1986).

2.4. Assessment of gene expression

RNA was extracted from cells using an RNeasy mini kit (Qiagen, Hilden, Germany). Reverse transcription was performed in a total volume of 20 μl using random hexamer primers (Applied Biosystems, Foster City, CA) and 0.5 μg total RNA. mRNA targets were amplified from cDNA by polymerase chain reaction (PCR) using an ABI/Prism 7700 Sequence Detector System. Each 25 μl reaction mixture contained 1 μl of cDNA, 12.5 μl of TaqMan Universal PCR Master Mix, 250 nM probes and 900 nM primers from the Assays-on-Demand Gene Expression Assay Mix (TaqMan, Applied Biosystems). The Assay-on-Demand probes used were cytochrome *c* oxidase subunit IV (COX4I1; Hs00266371), CEBPα (Hs00269972), lipoprotein lipase (Hs00173425), PPARγ (Hs00234592), adiponectin (Hs00605917), leptin (Hs00174877), adipocyte fatty-acid binding protein-4 (aFABP4/aP2; Hs00609791), sterol regulatory element-binding protein-1 (SREBP-1; Hs00231674) and 18S rRNA (Hs99999901). The sequences of primers and probe for the detection of cytochrome *c* oxidase subunit II (COII) and assessment of mitochondrial DNA (mtDNA) abundance, designed using the Assay-by-Design system (Custom TaqMan Gene Expression Assays, Applied Biosystems), were 5′-CAA ACC ACT TTC ACC GCT ACA C-3′ (forward primer), 5′-GGA CGA TGG GCA TGA AAC TGT-3′ (reverse) and 5′-AAA TCT GTG GAG CAA ACC-3′ (FAM-labeled probe). mtDNA was quantified using this primer/probe set and expressed relative to nuclear DNA, determined by amplification of the intronless gene C/EBPα. Controls containing no RNA, primers, or reverse transcriptase were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicates was used to calculate the relative amount of each target mRNA. The mean value for each target was normalized to that of the 18S rRNA gene using the comparative ($2^{-\Delta CT}$) method following the Manufacturer's instructions.

2.5. Assessment of adipokine and cytokine secretion

Adipokines and cytokines released by adipocytes were quantified using 25 μl of medium collected from adipocyte cultures corresponding to the last 5 days before harvest. Adiponectin, leptin, monocyte chemoattractant protein-1 (CCL-2), IL-6, IL-8, total plasminogen activator inhibitor type-1 (PAI-1), hepatocyte growth factor (HGF), and nerve growth factor (NGF) were detected using an antibody-linked, fluorescently labeled microsphere bead-based multiplex analysis system (Linco Research/Millipore, Billerica, MA) and quantified using Luminex100ISv2 equipment. Although the multiplex system used (HADCYT-61K, Millipore) also allowed for the quantification of IL-1β, resistin and tumor necrosis factor-α, the levels of these factors in adipocyte culture medium were below detection limits under all conditions tested. Lactate in the medium was measured spectrophotometrically (Roche, Sant Cugat del Vallés, Spain).

2.6. Assessment of endogenous reverse transcriptase activity

Cellular reverse transcriptase (RT) activity assay was performed as previously reported (Mangiacasale et al., 2003). Briefly, cells were lysed in ice-cold lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol). After three freeze-and-thaw cycles, cells were incubated for 30 min on ice and centrifuged for 30 min at 14,000 rpm at 4 °C. The supernatant containing the RT activity was aliquoted, quickly frozen in dry ice and stored at −80 °C. The protein concentration was determined by Bradford analysis. RT activity was tested using a MultiScribe TM Reverse Transcriptase (Applied Biosystems) in 20 μL reactions containing 10 ng of MS2 phage RNA (Roche Diagnostics), 30 pmol of MS2 reverse primer (see below) and substituting commercial RT with cell-free extract (12 μg total protein). Reaction mixtures were incubated at 25 °C for 10 min, 48 °C for 30 min followed by 5 min at 95 °C. A 2 μL volume from each reaction with forward (5′-TCCTGCTCAA-CTTCCTGTCGAG-3′) and reverse (5′-CATAGGTC AAACCTCCTAGGAATG-3′) MS2 primers were analyzed by quantitative PCR using SYBR green fluorescent dye (Applied Biosystems).

HT-29 adenocarcinoma cell line was used as positive control cells of RT activity (Mangiacasale et al., 2003). RT activity was measured in differentiating human adipocytes after 2- or 15-days induction of differentiation. The effects of antiretroviral drugs were assessed in 48-h treated differentiating human adipocytes.

2.7. Statistics

Where appropriate, statistical analyses were performed using Student's *t*-test. Differences with *P*-values <0.05 were considered statistically significant.

3. Results

3.1. Effects of nevirapine and efavirenz on human adipocyte viability and differentiation

Nevirapine was not cytotoxic to human adipose cells at any of the concentrations tested. At low concentrations (0.5 and 4 μM), efavirenz was not significantly cytotoxic, but at 20 μM caused extensive cell death, precluding further analysis of differentiation effects at higher efavirenz concentrations (data not shown).

A quantitative assessment of the effect of drug treatment on the extent of differentiation of human adipocytes compared to untreated controls (defined as 100%) is shown in Fig. 1A and B. Representative microscopic images depicting the effects of drug treatment on morphological changes associated with the acquisition of adipocyte morphology during *in vitro* differentiation are shown in Fig. 1C. Nevirapine at 0.5, 2, 4 and 10 μM did not affect morphological adipocyte differentiation; in contrast, efavirenz at a concentration of 1 μM (data not shown), 2 and 4 μM (the highest non-toxic concentration) significantly impaired differentiation (Fig. 1A). Exposure of differentiating cells to 20 μM nevirapine significantly increased adipocyte differentiation. Increasing the nevirapine concentration to 30 and 40 μM did not further modify adipogenesis. Measurement of lipid accumulation by quantification of eluted Oil Red O stain also revealed an inhibition of differentiation by efavirenz (1 (data not shown), 2 and 4 μM) whereas nevirapine did not significantly affect lipid accumulation at any of the concentrations tested (Fig. 1B).

3.2. Effects of nevirapine and efavirenz on gene expression in human adipocytes differentiating in vitro

An analysis of marker genes of adipocyte differentiation and metabolism showed that treatment of human adipocytes with nevirapine during differentiation did not modify gene expression except at the 20 μM concentration, which caused a significant increase in the mRNA levels of the adipogenesis master regulator genes SREBP-1, PPARγ and C/EBPα as well as adiponectin, leptin and lipoprotein lipase, marker genes of adipogenic differentiation and metabolism (Fig. 2). In contrast, efavirenz exerted the opposite effect, causing a dose-dependent reduction in the expression of adipogenesis marker genes, first evidenced as a significant decrease in SREBP-1 mRNA, PPARγ mRNA and leptin mRNA at 0.5 μM efavirenz. At higher doses of efavirenz, the reduction in the expression of these genes was more pronounced and there was also a significant decrease in adiponectin and lipoprotein lipase mRNA levels beginning at 2 μM, and also in C/EBPα and aFABP4/aP2 mRNA levels at 4 μM. Neither drug caused significant mitochondrial toxicity at any of the concentrations analyzed, as evidenced by the absence of changes in COIV (nuclear DNA-encoded) or COII (mtDNA-encoded) mRNA levels, or the relative levels of mtDNA.

3.3. Effects of nevirapine and efavirenz on the release of adipokines, cytokines and lactate by differentiating human adipocytes

The effects of nevirapine and efavirenz at concentrations representative of their opposing effects on human adipocytes (20 μM nevirapine and 4 μM efavirenz) were studied for their effects on the release of regulatory proteins and lactate by adipocytes (Fig. 3). Efavirenz caused a dramatic decrease in adiponectin and leptin release into the medium. In contrast, nevirapine did not alter leptin release and significantly increased adiponectin levels in adipose cell culture medium. Moreover, efavirenz profoundly increased the expression of the pro-inflammatory cytokines IL-6, CCL-2 and IL-8, whereas nevirapine did not significantly alter either CCL-2 or IL-8 levels, and significantly decreased the levels of IL-6.

With respect to other regulatory proteins released by adipocytes, nevirapine had no effect on total PAI-1 levels whereas efavirenz significantly increased it, and nevirapine significantly reduced HGF levels in adipocyte culture medium whereas efavirenz increased it. Neither drug had any effect on NGF release, and there were no differences among controls, nevirapine-treated and efavirenz-treated adipocyte cell cultures with respect to release of lactate into the medium.

3.4. Effects of nevirapine and efavirenz on human adipocyte endogenous reverse transcriptase activity

Mainly nevirapine but also efavirenz have been reported to exhibit anti-proliferative and pro-differentiating effects on several cell types, including tumor cells, through inhibition of endogenous

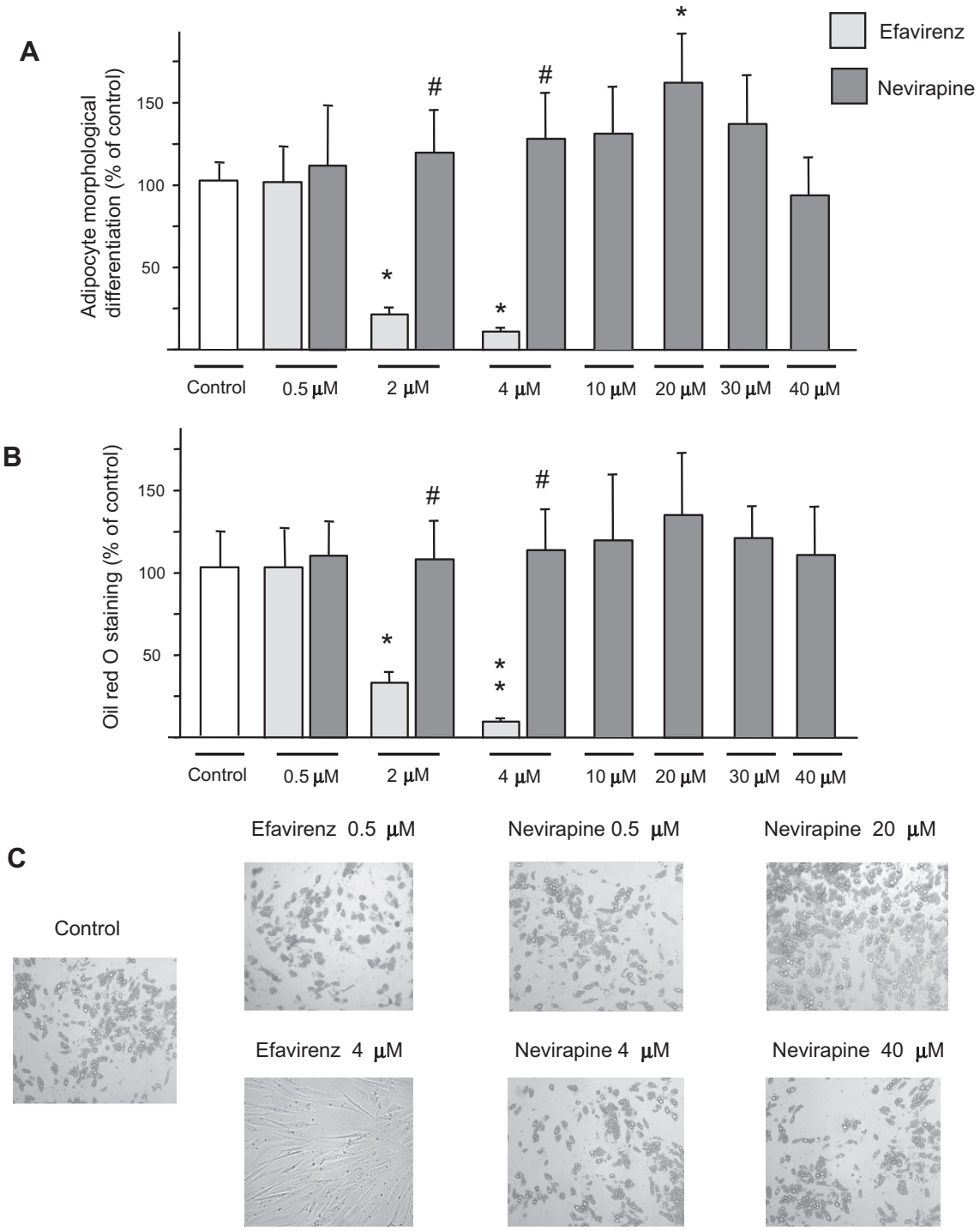

Fig. 1. Effects of nevirapine and efavirenz on human preadipocytes differentiating in culture. Human preadipocytes were differentiated in culture in the presence of the indicated concentrations of drugs. Bars are means ± SEM of the extent of morphological adipocyte differentiation (A) or of the Oil Red O staining of lipid accumulation (B) of 4–5 independent cultures at each of the indicated concentrations, and are expressed relative to values from untreated control cells, defined as 100% (see Section 2). ($^{*}P < 0.05$ and $^{**}P < 0.01$ for each drug treatment vs control; $^{\#}P < 0.05$ for nevirapine vs efavirenz treatment at the same concentration). (C) Representative microphotographs of adipocyte cell cultures differentiating in the presence of the indicated concentrations of drugs.

cellular reverse transcriptase (RT) activity (Spadafora, 2004). As depicted in Fig. 4A, quantitative assessment of endogenous RT activity showed higher levels in HT-29 adenocarcinoma cells (6-fold) and human preadipocytes (2-fold) than in human adipocytes, in agreement with more elevated endogenous RT activity in proliferating respect quiescent/differentiated cells in other cell types. When the effects of nevirapine and efavirenz were tested in human differentiating adipocytes, a different behavior was observed (Fig. 4B). Efavirenz had no effect on endogenous RT activity whereas nevirapine significantly reduced it, to levels similar to those found in fully differentiated human adipocytes.

4. Discussion

The present study establishes that nevirapine, in contrast to the other most widely used NNRTI, efavirenz, does not impair

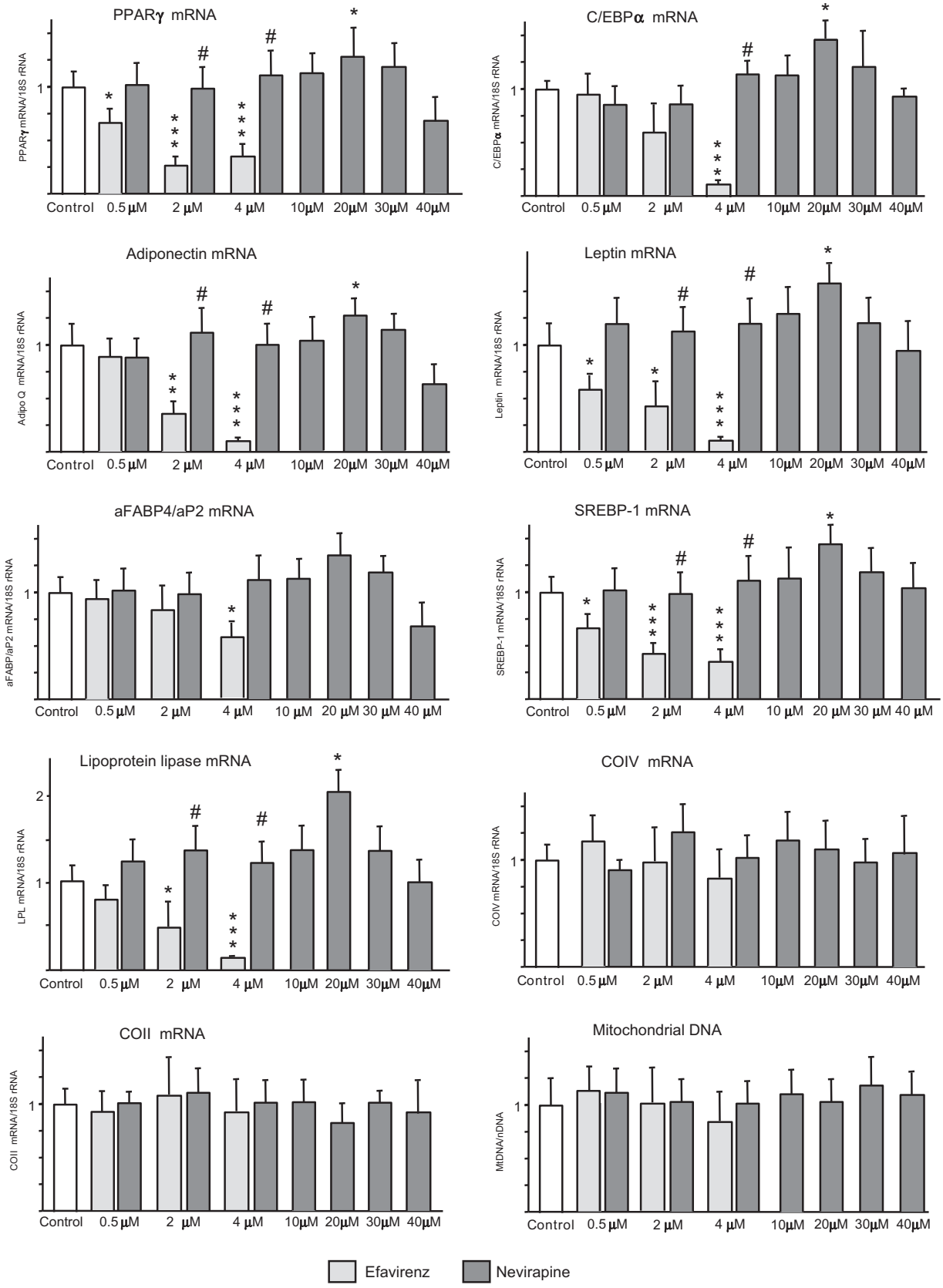

Fig. 2. Effects of nevirapine and efavirenz on mtDNA levels, and expression of genes related to mitochondrial function and adipogenic function in human adipocytes differentiating in culture. Human preadipocytes were differentiated in culture in the presence of the indicated concentrations of efavirenz (light gray bars) or nevirapine (dark gray bars). Data are presented as means ± SEM from 4 to 5 independent experiments, and are expressed relative to values from untreated control cells (defined as 1). ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for each drug treatment vs control; $^{\#}P < 0.05$ for nevirapine vs efavirenz treatment at the same concentration).

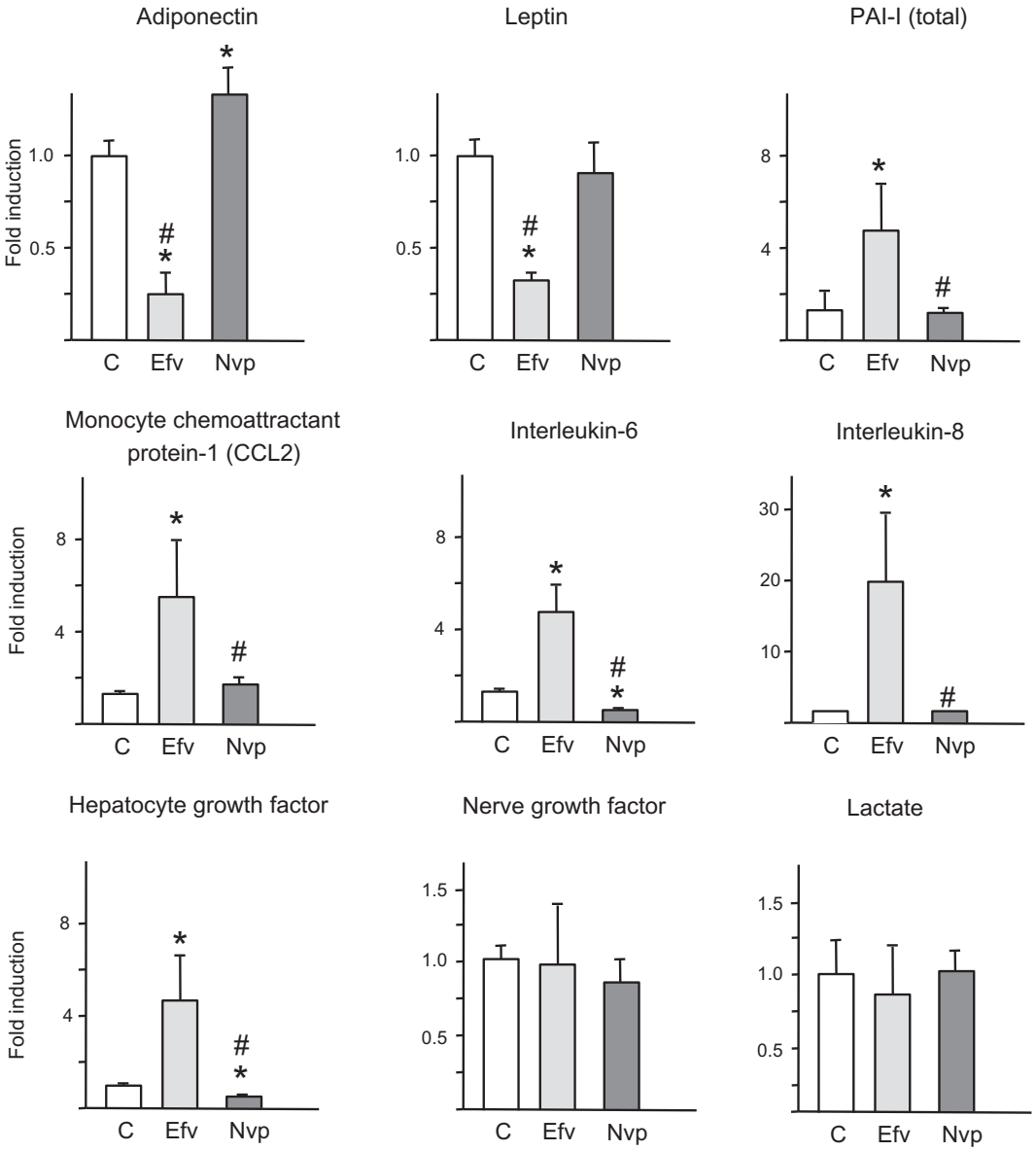

Fig. 3. Effects of nevirapine and efavirenz on the release of adipokines, cytokines and lactate by human adipocytes in culture. Human preadipocytes were differentiated in culture in the presence of 4 μM efavirenz (Efv) or 20 μM nevirapine (Nvp), and release of adipokines, cytokines and lactate was measured. Values represent concentrations in cell culture medium, presented as means ± SEM from 4 to 5 independent experiments and expressed relative to values from untreated control cells (defined as 1). Mean values of cytokines, adipokines and lactate in the medium from control untreated cells were as follows: adiponectin, 0.24 ± 0.3 μg/ml; leptin, 9.0 ± 0.8 ng/ml; total PAI-I, 0.41 ± 0.06 ng/ml; CCL-2, 3.2 ± 0.4 ng/ml; IL-6, 66 ± 9 pg/ml; IL-8, 29 ± 4 pg/ml; HGF, 85 ± 10 pg/ml; NGF 8.1 ± 1.1 pg/ml; lactate, 2.1 ± 0.5 mM. ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for each drug treatment vs control; $^{\#}P < 0.05$ for nevirapine vs efavirenz treatment).

adipogenesis and even causes mild positive effects at a single concentration. Negative effects of efavirenz on human adipocyte differentiation have been reported (El Hadri et al., 2004) and recent data from clinical trials indicate potentially negative effects of efavirenz when used as part of some antiretroviral drug combinations (Haubrich et al., 2009). In the present study, we confirmed that these detrimental effects of efavirenz are associated with a profound dose-dependent repression of the master transcriptional regulators of adipogenesis, SREBP-1, PPARγ and C/EBPα, and a reduction in the expression of genes involved in fat accretion in the adipose cells, such as lipoprotein lipase. These effects are in strong contrast to the effects of nevirapine, which did not cause inhibition of adipogenesis at any concentration tested, in agreement with lack of nevirapine effects on murine 3T3-F442A adipocyte differentiation (Caron et al., 2004). Present data also indicate that, in fact, at 20 μM nevirapine even tended to have the opposite effect, significantly increasing the morphological differentiation of adipocytes, and enhancing the expression of PPARγ and C/EBPα, and genes associated with the adipocyte phenotype, such as lipoprotein lipase and adiponectin.

Neither the lack or mild positive effects of nevirapine nor the negative effects of efavirenz on human adipocytes involved mitochondrial alterations. The absence of changes in mtDNA levels, expression of mtDNA-encoded or nuclear DNA-encoded transcripts for mitochondrial proteins, or lactate release into the medium indicates that NNRTIs may alter adipocyte biology without causing mitochondrial toxicity. This is in agreement with studies on adipose tissue from patients, which have shown that the most marked mitochondrial toxicity is attributable to the inclusion of NRTI thymidine-analogs as part of treatment, rather than to the

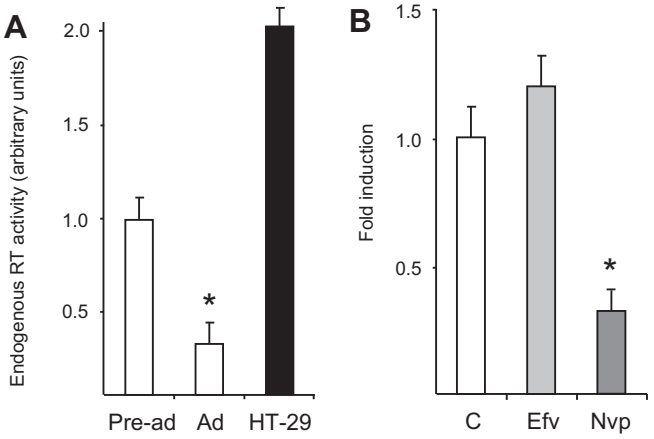

Fig. 4. Effects of nevirapine and efavirenz on human adipocyte endogenous reverse transcriptase activity. (A) Functional RT activity assay after incubation of MS2 RNA with lysates from human preadipocytes (Pre-ad), human adipocytes (Ad) and HT-29 adenocarcinoma cells. (*$P < 0.05$, for comparisons between Pre-ad vs Ad). (B) Human preadipocytes differentiating in culture were treated for 48 h in the presence of 4 μM efavirenz (Efv) or 20 μM nevirapine (Nvp), and endogenous RT activity was measured. (*$P < 0.05$, for drug treatment vs control).

presence of NNRTIs efavirenz or nevirapine (Villarroya et al., 2007b).

Notably, the present finding that nevirapine may favor the differentiation of adipose cells is consistent with multiple reports indicating positive effects of nevirapine on differentiation and growth arrest in other cell types. Nevirapine has been reported to cause differentiation of normal and multiple types of transformed cells (Mangiacasale et al., 2003), including human prostate carcinoma cells (Landriscina et al., 2009), renal carcinoma cells (Landriscina et al., 2008), cervical carcinoma cells (Stefanidis et al., 2008) and thyroid carcinoma cells (Landriscina et al., 2006; Modoni et al., 2007). This has led to proposals to explore the use of nevirapine in cancer treatment. However, the molecular mechanisms by which nevirapine promotes cell differentiation are currently uncertain, although inhibition of endogenous cellular reverse transcriptase activity has been proposed (Spadafora, 2004; Sciamanna et al., 2005). Present results indicate for the first time that inhibition of endogenous RT activity occurs during human adipocyte differentiation, and that nevirapine is capable to inhibit endogenous RT activity in differentiating preadipocytes. In contrast, although efavirenz was also showed to have inhibitory effects on endogenous reverse transcriptase as well as similar anti-proliferating and pro-differentiating effects than nevirapine on transformed cells (Sciamanna et al., 2005; Landriscina et al., 2008), efavirenz did not affect endogenous RT activity in human preadipocytes, consistently with its negative role on adipocyte differentiation. Thus, differential effects of nevirapine and efavirenz on adipocyte-specific reverse transcriptase activity, together with efavirenz-specific repression of master transcriptional regulators of adipogenesis (El Hadri et al., 2004; Esposito et al., 2009; Gallego-Escuredo et al., 2010, and present results), would constitute an additional mechanism by which nevirapine and efavirenz have differential effects in the context of adipose cells.

The beneficial effects of nevirapine on human adipose cells – from promoting adipogenesis to attenuating the release of pro-inflammatory molecules and stimulating the release of insulin sensitizing, anti-inflammatory molecules such as adiponectin – were evident at only at 20 μM, suggesting a narrow optimal concentration range for these effects. This is similar to our previous findings obtained using the murine brown adipocyte cell culture model (Rodríguez de la Concepción et al., 2005). In addition to its anti-adipogenic effects, efavirenz induced the release of pro-inflammatory cytokines (e.g., CCL-2, IL-6 and IL-8) and other molecules related to the inflammatory processes, such as PAI-1 and HGF. Efavirenz also decreased the release of adiponectin into the medium. In contrast, nevirapine favored the release of adiponectin and repressed the release of IL-6 and HGF. Adiponectin is recognized as a major insulin-sensitizing hormone with anti-inflammatory properties (Kadowaki et al., 2006), and low levels of serum adiponectin are commonly found in HIV-1-infected patients with lipodystrophy and/or metabolic syndrome (Tong et al., 2003). The relevance of these observations in relation to the metabolic status of HAART-treated patients should deserve further attention. Some studies have indicated that switching to nevirapine reduces hyperinsulinemia (Domingo et al., 2001), whereas other reports have found that switching to nevirapine has no effect on adiponectin levels or insulin sensitivity, despite normalization of the lipid profile (Petit et al., 2004). The fact that 20 μM nevirapine decreased the release of IL-6, a pro-inflammatory cytokine that plays a main role in adipose tissue signaling being responsible for eliciting systemic insulin resistance (Kim et al., 2009), is notable, as is the nevirapine-induced decrease in the release of HGF, a powerful angiogenic factor produced by adipose tissue. HGF is increased in the obesity-induced inflammation of adipose tissue (Bell et al., 2008), but data on possible changes in HGF in HIV-1-infected patients undergoing HAART are currently lacking. Collectively, these observations lead us to hypothesize that the presence of nevirapine in HAART cocktails may moderate the local pro-inflammatory environment in adipose tissue elicited by infection-related events and concurrent treatment with other drugs. This might be especially relevant in light of growing evidence that local inflammation in adipose tissue plays a role in mediating systemic metabolic disturbances in patients (Villarroya et al., 2007a), and should drive further attention in clinical studies comparing the side effects of individual drug components of HAART regimes.

In summary, the present results suggest that nevirapine does not inhibit adipogenesis and may even exert mild positive effects on the pattern of inflammation-related signals released by human adipocytes. This is in contrast to the profound anti-adipogenic and pro-inflammatory pattern of response elicited by efavirenz. The present *in vitro* adipocyte study has obvious limitations in terms of extrapolation to the treatment of patients. Notably, however, the average nevirapine plasma concentration in patients is approximately 20 μM (Dupin et al., 2002; Cooper and van Heeswijk, 2007), suggesting that the beneficial effects of nevirapine observed *in vitro* may be relevant *in vivo*. Complicating this interpretation is the fact that serum proteins may bind substantial amounts of drug; thus, the actual free nevirapine concentration in blood may be lower than that in serum-free adipocyte culture medium containing 20 μM nevirapine. However, because nevirapine has been reported to accumulate in adipose tissue from patients, reaching a concentration much higher than that in blood (Dupin et al., 2002), the possibility that adipocytes *in vivo* are exposed to local concentrations of nevirapine that approach the 20 μM range cannot be excluded. The same rationale applies to efavirenz concentrations, as this drug also accumulates at much higher concentrations in adipose tissue than blood of treated patients (Dupin et al., 2002). In any case, because nevirapine is already widely used in certain settings (e.g., some European countries, developing world) and given the potential deleterious metabolic side-effects of antiretroviral treatments, any evidence of neutral, or maybe beneficial, effects of nevirapine should be considered in the context of optimizing drug composition in HAART. However, the fact that nevirapine has been associated with selection of resistant virus as well as with life-threatening hepatic and cutaneous toxicities (de Béthune, 2010) should also be taken into consideration.

Acknowledgements

The study was supported by Grants from Ministerio de Ciencia e Innovación (SAF2008-01896), Fondo de Investigaciones Sanitarias (PI08-1715) and Red de Investigación en SIDA (RD06/006/0022), Instituto de Salud Carlos III and FIPSE (36610/06), Spain, and an independent grant from Boehringer Ingelheim. This company had no role in the study design, data collection, interpretation of data or manuscript preparation.

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Differential molecular signature of visceral adipose tissue alterations in HIV-1-associated lipodystrophy

Revista: AIDS (enviado)

Titulo: características moleculares diferenciales de las alteraciones del tejido adiposo visceral de pacientes infectados por el HIV-1 con lipodistrofia.

La lipodistrofia en pacientes infectados por el virus HIV-1 se asocia comúnmente a alteraciones opuestas en los diferentes depósitos de tejido adiposo, por ejemplo la lipoatrófia a nivel subcutáneo contra la lipohipertrofia a nivel visceral. En este artículo describimos las alteraciones específicas del tejido adiposo visceral respecto al subcutáneo. Para ello analizamos la expresión de marcadores de función mitocondrial, adipogénesis e inflamación de 8 biopsias de grasa omental de pacientes infectados por el HIV-1 con lipodistrofia para compararlo con biopsias de tejido adiposo subcutáneo de otros 10 pacientes y muestras de tejidos adiposos subcutáneos y visceral de controles sanos. La depleción de DNA mitocondrial y el aumento de proteína mitocondrial se han hallado similares en ambos tejidos para los pacientes. Los transcritos de marcadores de adipogénesis y metabolismo se han visto inalterados en el tejido adiposo visceral pero disminuidos en el subcutáneo de los pacientes respecto a controles sanos. TNFα y CD68 están inducidos en ambos tejidos de los pacientes pero otros marcadores de rutas de inflamación presentan alteraciones distintas en el tejido adiposo visceral de los pacientes. La IL18 e IL1RN están inducidas solo en el tejido adiposo subcutáneo, en cambio las interleuquinas 6 y 8, así como MCP1 aparecen reducidas en el tejido adiposos visceral pero no en el subcutáneo de los pacientes. Se puede concluir que las alteraciones mitocondriales suceden de manera similar en los tejidos adiposos visceral y subcutáneo de los pacientes. En cambio la expresión de genes adipogénicos no se ve alterada en el visceral de estos pacientes marcando este hecho como un proceso importante en las alteraciones diferentes que observamos en los dos depósitos de los pacientes. Las alteraciones específicas que se observan en ambos tejidos a nivel inflamatorio, siendo más suave la señalización pro-inflamatoria en el tejido visceral, podrían contribuir a este comportamiento opuesto que presentan ambos tejidos en pacientes.

Differential molecular signature of visceral adipose tissue alterations in HIV-1-associated lipodystrophy

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Running title: HIV-1 lipodystrophy and adipose depots

Keywords: lipodystrophy, subcutaneous adipose tissue, visceral adipose tissue, mitochondria, adipokines, adipocyte differentiation, inflammation

Word count: 3499 words

Supported by grants: SAF2011-23636 from Ministerio de Ciencia e Innovación, Spain; PI08/1715 and PI11/00376 from Fondo de Investigaciones Sanitarias and RD06/0006/0022 from Red de Investigación en SIDA, Instituto de Salud Carlos III, Spain; and 2009-SGR284 from AGAUR-Generalitat de Catalunya.

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ABSTRACT

Objective: Lipodystrophy in HIV-1-infected, antiretroviral-treated-patients is often associated with opposite alterations in adipose tissue depots, i.e. lipoatrophy of subcutaneous (SAT) versus lipohypertrophy of visceral (VAT) adipose tissues. We determined the specific alterations in VAT respect to SAT in patients.

Design: We analyzed the expression of marker genes of mitochondrial function, adipogenesis and inflammation in a unique collection of eight biopsies of omental VAT from HIV-1-infected, antiretroviral-treated patients, with lipodystrophy. For comparison, SAT from ten patients, and SAT and VAT from ten non-infected individuals were analyzed.

Methods: Quantitative real-time PCR of mitochondrial DNA and gene transcripts; immunoblot and multiplex for quantification of specific proteins.

Results: Mitochondrial DNA depletion and abnormal increases in mitochondrial protein levels were similarly found in VAT and SAT from patients. Transcript levels of marker genes of adipogenesis and metabolism were unaltered in VAT but lowered in SAT. TNFα and CD68 were similarly induced in both adipose depots from patients, but other markers of inflammation-related pathways showed distinct alterations: interleukin-18 and IL1RN were induced only in SAT whereas expression of interleukin-6, –8 and MCP-1 was reduced in VAT but not in SAT.

Conclusions: Mitochondrial alterations occur similarly in VAT and SAT from patients. Adipogenic gene expression is unaltered in VAT from patients, highlighting the relevance of the adipogenic processes in the differential alterations of fat depots. Specific disturbances in inflammatory status in VAT respect to SAT are present. Milder induction of pro-inflammatory signaling in VAT could be involved in the preservation from fat wasting in this depot.

INTRODUCTION

The lipodystrophy syndrome is one of the major disturbances occurring in HIV-1-infected patients, with prevalence rates around 40-50% [1]. It is characterized by a complex set of alterations in adipose tissue: mainly, peripheral lipoatrophy (that is a severe loss of subcutaneous adipose tissue in the face, arms, and legs) which is often accompanied by visceral lipohypertrophy (that is an enlargement in visceral adipose tissue from the abdomen, and breast in women). Less prevalent is the occurrence of lipomatosis, which appears usually as an enlargement in the dorso-cervical area, the so-called 'buffalo hump' [1,2]. Although these alterations do not necessarily occur altogether, a same patient may show lipoatrophy in the face and arms and enlarged adiposity in the visceral area. In addition, HIV-1 patients with lipodystrophy show metabolic alterations reminiscent of the metabolic syndrome, particularly dyslipidemia and insulin resistance. This alterations lead to enhanced cardiovascular risk in patients and favor the development of diabetes. Despite the fact that the trigger of the syndrome is a complex combination of HIV-1-infection and drug treatment-related events, inflammation-related events and lipotoxicity appear as likely mechanism for the development of the syndrome [3,4]. Recently, as a consequence of the development of novel antiretroviral drugs with lower toxicity, overt peripheral lipoatrophy has diminished its frequency. However, visceral lipohypertrophy and associated metabolic alterations remain a common concern in treated patients [5].

In order to understand the etiopathogenesis of lipodystrophy in HIV-1-patients, several studies have analyzed changes in gene expression appearing in adipose tissue of patients but, practically all studies have been performed using biopsies obtained from subcutaneous fat depots, that is an adipose depot suffering a lipoatrophic alteration. The results of such studies established that there was impaired content of mitochondrial DNA, altered expression of mitochondrial DNA-encoded genes, reduced

expression of master regulators of adipogenesis and their targets as well as up-regulation of genes involved in inflammation in adipose tissue from patients showing full-blown lipodystrophy [6-9]. Furthermore, the study of patients of different status in relation to HIV-1 infection, antiretroviral treatment and appearance of lipodystrophy suggested that some alterations in adipose tissue gene expression occur as a consequence of infection whereas other disturbances are more related to treatment or appear only in association with the establishment of full-blown lipodystrophy [8].

Whatever its etiology, the lipodystrophy syndrome can be considered a consequence of adipocyte dysfunction. However, changes in subcutaneous (lipoatrophy) versus visceral (lipohypertrophy) adipose tissues are opposite. Until present, no data is available on the specificity of molecular alterations in visceral fat from HIV-1 patients leading to lipohypertrophy, surely due to the difficult availability of such adipose samples. Here we analyzed mRNA and protein expression of several markers of mitochondrial function, adipogenesis and inflammation in a unique collection of intra-abdominal visceral (omental) adipose tissue (VAT) biopsies from HIV-1-infected, HAART-treated patients, with lipodystrophy. This specific profile of marker gene expression was compared to that of abdominal subcutaneous adipose tissue (SAT) from patients, as well as to those of SAT and VAT from non-infected control individuals.

METHODS

All patients and controls provided informed written consent to participate in the study. The study was approved by the Ethics Committee of Hospital de la Santa Creu i Sant Pau, Barcelona. Patients with opportunistic infections, neoplasms, or fever of undetermined origin were excluded from the study. At the time of the study entry, no

patient or control used any other drug known to influence glucose metabolism or fat distribution, such as anabolic hormones or systemic corticosteroids, uridine, recombinant human growth hormone or appetite stimulants or suppressors. Biopsy samples of subcutaneous adipose tissue from controls and patients showing lipodystrophy were taken from the abdominal area. Samples of omental (visceral) adipose tissue were obtained on occasion of laparoscopic cholecystectomy. Tissue samples were immediately frozen in liquid nitrogen. After homogenization in RA1 buffer (Macherey-Nagel, Düren, Germany), an aliquot was used for isolation of DNA, which was performed using a standard phenol/chloroform extraction methodology. RNA was obtained using a column-affinity based methodology (NucleoSpin, Macherey-Nagel) and included on-column DNA digestion (rDNase, Macherey-Nagel). One half μg of RNA was transcribed into cDNA using MultiScribe reverse transcriptase and random-hexamer primers (TaqMan Reverse Transcription Reagents, Applied Biosystems, USA). For quantitative mRNA expression analysis, TaqMan RT-PCR was performed on the ABI-PRISM 7700HT sequence detection system (Applied Biosystems, USA). The TaqMan RT-PCR reaction was performed in a final volume of 25 μl using TaqMan Universal PCR Master Mix, No AmpErase UNG reagent and the specific gene expression primer pair probes (Applied Biosystems, USA).

The Assay-on-demand probes (TaqManGene Expression Assays, Applied Biosystems, USA) used were: 18S rRNA, Hs99999901_s1; CEBPA, Hs00269972_s1; LPL, Hs00173425_m1; TNF, Hs00174128_m1; PPARG, Hs00234592_m1; Adiponectin, Hs00605917_m1; CD68, Hs00154355_m1; SLC2A4 (GLUT4), Hs00168966_m1; IL6, Hs00174131_m1; IL8, Hs00174103_m1; IL18, Hs99999040_m1; IL1RN , Hs00893625_m1; CCL2 (MCP1), Hs00234140_m1; CYCS, Hs01588973_m1; MT-CYB, Hs02596867_s1; COX4I1, Hs00266371_m1; MT-COII, Hs02596865_g1. Quantification of mtDNA was performed using cyt-b prove (MT-CYB)

and referred to nuclear DNA, as determined by the amplification of the intronless gene CEBPα (CEBPA). Appropriate controls with no RNA, primers, or reverse transcriptase were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA expression of the genes of interest which were normalized to that of the reference control (18S ribosomal RNA) using the comparative ($2^{-\Delta CT}$) method, following the manufacturer's instructions. Parallel calculations using the PPIA (Hs99999904_m1) and HPRT1 (Hs99999909_m1) reference genes were performed and results were essentially the same.

For quantification of protein levels, adipose tissue samples were homogenized in cold buffer (10 mM HEPES pH 7.5, 5 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$), and a cocktail of protease inhibitors (Complete-mini, Roche, Spain). For Western blot analysis, 40 μg of homogenate protein were mixed with equal volumes of 2 x sodium dodecyl sulphate (SDS) loading buffer, incubated at 90ºC for 5 min, and electrophoresed on SDS/polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA) and immunological detection was performed using antibodies directed against SREBP1 (K-10, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β_2-microglobulin (Dako, Glostrup, Denmark), COIV (Molecular Probes, Leiden, Netherlands), and total OXPHOS (MitoSciences, Oregon, USA). Goat anti-mouse and anti-rabbit HRP-conjugate antibodies (Bio-Rad, Hercules, CA, USA/Santa Cruz Biotechnology) and ECL reagents (Immobilon Western, Millipore) were used to detect the immunoreactive signals. Coomassie blue (Sigma-Aldrich) staining of the membranes was used to normalize the amount of protein loaded. Multi-Gauge software (Fujifilm) was used for densitometric analyses. Monocyte chemoattractant protein-1/CCL-2 (MCP-1), interleukin-6 (IL-6), interleukin-8 (IL-8), total plasminogen activator inhibitor type-1 (PAI-1), and hepatocyte growth factor (HGF) were quantified in adipose tissue extracts using a multiplex system (Milliplex

human adipokine, Linco Research/Millipore, Saint-Charles, MO, USA) and the Luminex100ISv2 equipment.

The median and the 25th-75th percentiles (interquartile range; IQR) were used to describe non-normally distributed quantitative data, otherwise the mean with standard deviation was reported. Where appropriate, either analysis of variance or Student's t-tests were used to determine significance of differences between groups. The analysis was performed using the Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, Illinois,USA) and the SAS version 9.1.3 software (SAS Institute Inc., Cary, North Carolina, USA), and the level of significance was established at the 0.05 level (two-sided).

RESULTS

Demographics, treatment data, anthropometric and biochemical parameters in patients and controls are shown in Table 1. Patient's groups were no significantly different in age, gender and indicators of overall adiposity (BMI, WHR). For the two HIV-1-infected groups of patients, no parameter related to cumulative antiretroviral drug treatment, systemic metabolic parameters or viral load indicators differed significantly. However, HIV-1 patients showed significantly higher levels of blood glucose and triglycerides, as well as higher levels of HOMA-r, indicative of insulin resistance. The patient groups showed peripheral lipoatrophy, in accordance with objective scales as reported by Fontdevila *et al.* [10].

The alterations related to adipose tissue mitochondrial toxicity are depicted in Figure 1. Mitochondrial DNA levels were significantly higher in visceral versus subcutaneous adipose tissue in healthy controls. In HIV-1-infected patients, there was a significant depletion in mtDNA levels in both types of adipose depots (Fig 1A),

although mtDNA content remained significantly higher in visceral than in subcutaneous fat. For mtDNA-encoded transcripts, both cytochrome b (cyt b) mRNA and the subunit II of the cytochrome oxidase (COII) mRNA were significant reduced only in subcutaneous fat and not in visceral fat from patients (Fig 1B). However, nuclear-encoded cytochrome c (cyt c) and subunit IV of the cytochrome oxidase (COIV) transcripts were significantly lowered in both subcutaneous and visceral adipose tissue from patients. Furthermore, we analyzed how mitochondrial toxicity translates to alterations in mitochondrial proteins (Fig 1C). We observed that both types of adipose tissue depots from patients showed increased levels of several mitochondrial proteins, both nuclear-encoded (ATPsynα) or mtDNA-encoded (COII). However, these alterations were not common to all mitochondrial proteins analyzed and, for instance, complex II FeS-protein levels were unchanged in any fat samples from patients versus controls. For COIV protein a significant increase was observed only in visceral fat from patients. In summary, adipose tissue from patients evidenced profound alterations in mitochondrial DNA, transcript and protein levels, in accordance with the overall mitochondrial toxicity often associated with lipodistrophy. The present data establish, however, that for most of the parameters analyzed, alterations in visceral adipose tissue are similar to those in subcutaneous fat, and only minor changes appear differentially in the omental depot from patients.

Next, we analyzed the alterations in the expression of marker genes of adipogenesis. In accordance with previous reports, the expression of the master gene for adipogenesis PPARγ, as well as its metabolic targets involved in glucose uptake (Glut4) and fatty acid uptake (lipoprotein lipase, LPL) were significantly repressed in subcutaneous fat from patients (Fig 2A). The same was observed for the adipokine adiponectin. Visceral adipose tissue from controls expressed lower transcript levels for some of these genes (LPL, Glut4) respect to control subcutaneous fat, and, in none of

the genes analyzed, visceral fat from patients showed any difference respect to visceral control fat, quite distinctly from the observations in subcutaneous fat. We extended the study of the SREBP1 protein, another master regulator of adipogenesis for which enhanced protein levels have been reported in subcutaneous fat from HIV-1-patients with lipodystrophy [6]. Effectively, SREBP1 protein levels in subcutaneous adipose tissue were significantly higher in patients than in controls. In contrast, no significant difference was found for SREBP1 protein in visceral fat from patients. (Fig 2B).

We next determined the profile of alterations in marker genes of inflammation, an alteration known to take place in subcutaneous fat from patients. We observed that subcutaneous fat from patients showed high levels of the pro-inflammatory cytokine TNFα respect to healthy controls. The same was observed when visceral fat from controls and patients was compared. Parallel alterations were found for the transcript levels of CD68, a marker of infiltrating macrophages. Despite these common alterations in subcutaneous and visceral fat from patients, further analysis to a more expanded set of marker genes related to inflammatory signaling indicated a differential behavior in visceral versus subcutaneous fat in patients. Thus, interleukin-18 was dramatically induced in subcutaneous fat from patients but unaltered in visceral fat. A similar pattern of changes was found for interleukin-1 receptor antagonist (IL1RN) transcript levels. Conversely, interleukin-6, interleukin-8 and MCP-1 transcripts were unaltered in subcutaneous fat from patients but significantly down regulated in visceral fat. It is worth mentioning that these transcripts were much more highly expressed in visceral than in subcutaneous fat from healthy controls. To get insight in the differential inflammation-related alterations in visceral adipose tissue from patients, we analyzed changes in several representative proteins. First we found that levels of β2-microglubulin protein, which gene is a target of TNFα [11], were similarly increased in

subcutaneous and visceral fat from patients. In contrast, interleukin-6, -8 and MCP-1 protein levels showed totally distinct alterations in the two adipose depots. As for the corresponding transcripts, protein levels for these cytokines were much higher in visceral than in subcutaneous fat from healthy controls. In subcutaneous fat from patients, there were no alterations or even a mild induction (MCP-1) in the levels of these cytokines. Conversely, interleukin-6, -8 and MCP-1 levels were dramatically reduced only in visceral adipose tissue from patients. This pattern of alterations was not common to all inflammation-related proteins. For instance, the levels of PAI-1, a natural inhibitor of fibrinolysis produced by adipose tissue among other tissues and which plasma levels are increased in patients with HIV-1/HAART-associated lipodystrophy [12], were similarly reduced in patient's adipose tissue samples either subcutaneous or visceral. The levels of HGF, an angiogenic factor known to be released by adipose tissue under pro-inflammatory conditions [13], were not significantly altered in any adipose tissue depot from patients respect to controls.

DISCUSSION

In the present study, we provide for the first time a comparative characterization of the alterations in the molecular signature of visceral adipose tissue in HAART-treated, HIV-1-infected patients with lipodystrophy, in comparison with the alterations occurring in subcutaneous adipose tissue.

Mitochondrial toxicity is claimed to be a relevant insult leading to some features of lipodystrophy, especially peripheral lipoatrophy [14]. Collectively, the present data indicate that most of the alterations indicative of mitochondrial toxicity occur similarly in visceral and subcutaneous fat, despite their opposite gross modifications in HIV-1-lipodystroply (lipohypertrophy versus lipoatrophy, respectively). As a general trend,

mitochondrial DNA depletion, lowered expression of some transcripts for mitochondrial proteins, and reactive increase in mitochondrial protein levels, all them alterations commonly found in subcutaneous fat from patients [7,8,15-17, and present findings], appear to take place similarly in visceral fat. These findings suggest that mitochondrial alterations are unlikely to determine the opposite behavior of the two fat depots in response to viral and drug-induced insults. However, specific decrease in mt-DNA encoded transcripts together with lower mtDNA content in subcutaneous fat is likely to result in a higher mitochondrial dysfunction in subcutaneous than in visceral fat. This has been observed in zidovudine-treated rats, in which specific decreases in mtDNA content and in mitochondrial function were found in subcutaneous but not in visceral fat depots [18]. Otherwise, higher mitochondrial content of visceral adipose tissue may account for a better adaptive compensatory reaction to altered mitochondrial function [4,19]. In fact, progressive substitution of drugs with a high mitochondrial toxicity (i.e. thymidine analogs) by less mitochondrial damaging ones in treatment recommendations has resulted in amelioration of the subcutaneous fat atrophy signs but not in visceral hypertrophy and induction of systemic metabolic syndrome [4,17,20].

In contrast, the pattern of alterations in gene expression in relation to adipogenesis was dramatically different in visceral and subcutaneous adipose tissue from patients, both in what concerns molecular controllers of cellular adipogenesis (PPARγ, SREBP1), as well as transporters (Glut4) and enzymes (LPL) involved in metabolic accretion processes. A profound repression in the expression of these genes as well as abnormal induction of unprocessed SREBP1 protein has been reported to take place in subcutaneous fat from patients [6,21], as we confirmed in the present findings. However, visceral adipose tissue was totally refractory to these alterations. This is consistent with the lack of atrophy of this depot and highlights the relevance of

the adipogenic differentiation processes in eliciting the differential alterations of fat depots in patients.

One of differences between visceral and subcutaneous adipose tissue most commonly reported by multiple studies on metabolic diseases (e.g. obesity) is the extent of induction of a pro-inflammatory environment. In healthy individuals, the expression of pro-inflammatory cytokines such as IL-6 or MCP-1 is higher visceral than in subcutaneous adipose tissue [22,23]. These observations are confirmed in our present set of healthy controls when both adipose depots were compared. Moreover, pro-inflammatory insults in fat, such as in obesity, take place preferentially in visceral adipose tissue and, in fact, inflammation in visceral fat has been proposed to be especially relevant to the enhanced unhealthy metabolic disturbances in patients with visceral obesity [24,25]. Our present findings indicate that visceral and subcutaneous adipose tissue from patients share alterations indicative of a parallel induction of a pro-inflammatory environment (i.e. similar induction of TNFα expression, indications of parallel induction of macrophage recruitment). However, there are dramatic differences in the alterations in specific subsets of inflammation-related pathways, and this is strongly suggestive of a differential inflammatory response to the HIV-1-infection, HAART treatment-related insults in the two adipose depots. In visceral fat from patients, the repression in typical pro-inflammatory cytokines such as interleukin-6, -8 and MCP-1, confirmed at the transcript and protein level, in association with prevention from IL-18 induction lead to the intriguing conclusion of a much more moderate, and even impaired, pro-inflammatory reaction in visceral relative to subcutaneous fat. The preferential induction in subcutaneous fat of anti-inflammatory cytokines such as IL1RN could also be a reactive sign to this enhanced exposure to pro-inflammatory cytokines in this adipose depot, similarly to what has been reported to happen in other conditions of pro-inflammatory insults in fat, such type II diabetes and obesity [26,27]. Moreover,

the induction of IL1RN expression in subcutaneous adipose tissue from patients is consistent with previous observations indicating the simultaneous recruitment of M2-type macrophages (reparative, anti-inflammatory) and M1-type macrophages (pro-inflammatory) in this adipose depot [28].

It is tempting to speculate that systematic induction of most pro-inflammatory cytokines in subcutaneous adipose tissue is associated with wasting processes leading to atrophy, whereas much milder alterations in visceral adipose tissue preserve it from wasting. In fact, if, as often proposed [29,30], initial induction of intense local inflammatory signaling may be responsible for impairment in adipogenic processes (via repression of PPARγ by pro-inflammatory cytokines, for instance) in lipoatrophic subcutaneous fat, a milder inflammation in visceral fat could account for the present observations of preserved visceral adipogenic processes. These considerations may appear, at a first glance, contradictory with the well established prominent role of inflammation in visceral fat in other metabolic pathologies such as obesity [31]. However, it should be taken into account that there is a totally different paradigm underlying the causes of inflammation in obesity and in HIV-1 lipodystrophy: in obesity, inflammation is caused by intrinsic alterations in adipose tissues caused by fat hypertrophy elicited by positive energy balance whereas in HIV-1 lipodystrophy, external insults (HIV-1 infection, antiretroviral drugs) impact on non previously enlarged adipose tissue to promote inflammation. It may also happen that the signs of inflammation in visceral fat from patients (TNFα and CD68 induction) were just the indirect consequence of mild visceral obesity in these patients. On the other hand, although there are some evidences that local inflammation is among the earliest events taking place in subcutaneous fat from patients during the development of lipodystrophy, even before overt lipoatrophy [8], it cannot be discarded that lipoatrophy itself enhances the induction of pro-inflammatory pathways in subcutaneous adipose tissue

and, in visceral adipose tissue, the lack of atrophy prevents from some features of enhanced inflammation taking place in the subcutaneous depot. Observations such as the dramatic induction of IL-18, a cytokine highly involved in apoptotic processes [32], only in subcutaneous fat from patients is consistent with the notion that some of the pro-inflammatory signaling induction in SAT may be directly associated to the occurrence of the lipoatrophic process.

Several studies have compared previously the atrophic versus hypertrophic alterations in subcutaneous adipose tissue from patients by the analysis of dorso-cervical enlarged fat (“buffalo hump”) developing in some HIV-1 patients [21,33]. It is worth mentioning that, despite this dorsal fat depot is not visceral fat, enlargement is associated with mitochondrial DNA deletion similar to lipoatrophic subcutaneous fat from patients, preserved expression of adipogenic genes and absence of induction of pro-inflammatory signaling. These observations are consistent with present findings in the sense that overt inflammation appears to be closely associated to a lipoatrophic status in adipose tissue from HIV-1 patients, regardless of anatomical placement.

Several limitations affect the extent of the conclusions that can be drawn from the present study. The low number of samples, specially the limited availability of omental adipose tissue from HIV-1 patients, limits the capacity to unequivocally clarify issues such as the role of treatment drug patterns or the actual significance of gene expression data when differences between groups lack statistical significance. Moreover, the lack of availability of biopsies from visceral and subcutaneous adipose tissue from the same individuals also precludes a proper assessment of the impact of individual variability on gene expression patterns in adipose tissue. Finally, the present study did not undertake the direct discrimination on the cellular origin of the alterations in the profile of gene expression for cytokines in adipose tissues, most of which in addition to being expressed by adipocytes, are intensely expressed and released by infiltrating macrophages and even T-lymphocytes in adipose depots. Despite these

limitations, the present results constitute the first systematic analysis of gene expression in visceral adipose tissue from HIV-1-infected patients on HAART and reveal a markedly differential pattern of alterations in visceral fat with respect to subcutaneous fat of patients showing lipodystrophy. The knowledge on the alterations of visceral adipose tissue in patients, given the prominent role of this fat depot in the control of systemic metabolic alterations, may help to envisage treatment and prevention strategies for a metabolic-friendly approach of HIV-1 patient's treatment.

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FIGURE LEGENDS

Figure 1

Mitochondrial DNA abundance and expression of mitochondrial function marker genes in subcutaneous (SAT) and visceral (VAT) adipose tissues from HIV-1-infected patients with lipodystrophy (LD), compared with adipose tissues from healthy controls.

The figure shows means ± SEM expressed in arbitrary units for mtDNA content (ratio relative to nuclear DNA) (A), and for each specific mRNA quantification (using 18S rRNA for normalization) (B). Representative immunoblot images are shown and bars are means ± SEM for each group, expressed in densitometric arbitrary units (C). Statistical significance ($P<0.05$) of differences between patients and Controls is shown as *, and between subcutaneous (SAT) and visceral (VAT) controls or patients is shown as #.

Figure 2

Expression of adipogenic marker genes in subcutaneous (SAT) and visceral (VAT) adipose tissues from HIV-1-infected patients with lipodystrophy (LD), compared with adipose tissues from healthy controls (C).

The figure shows means ± SEM expressed in arbitrary units for each specific mRNA quantification (A). 18S rRNA levels were used to normalize results. A representative protein immunoblot image is shown and bars are means ± SEM for each group, expressed in densitometric arbitrary units (B). Statistical significance of differences between patients and controls is shown as *, and between subcutaneous (SAT) and visceral (VAT) controls or patients is shown as #.

Figure 3

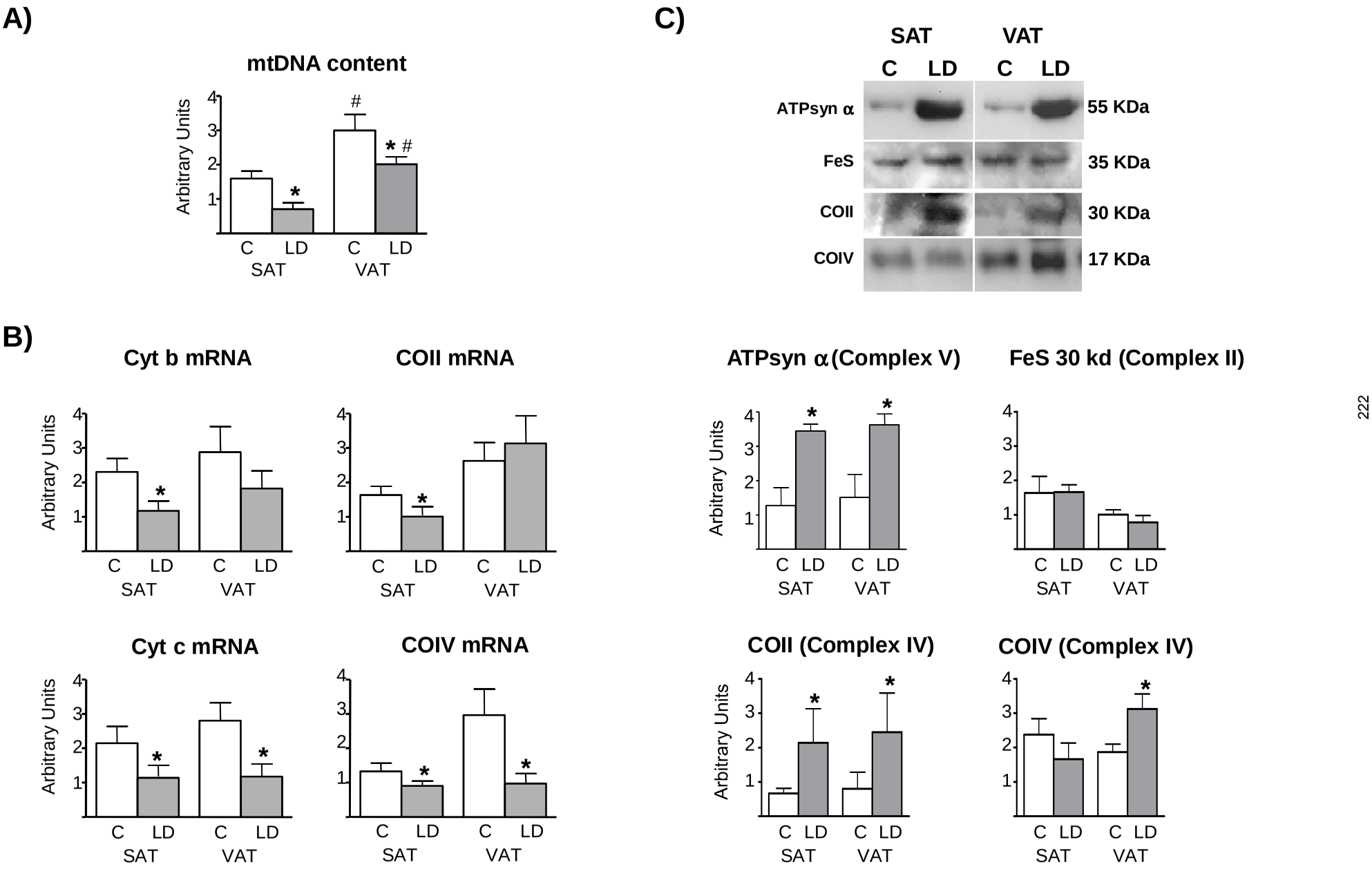
Expression of inflammation marker genes in subcutaneous (SAT) and visceral (VAT) adipose tissues from HIV-1-infected patients with lipodystrophy (LD), compared with adipose tissues from healthy controls.

Levels of mRNAs (A) and proteins (B) are shown in the figure. Bars are means ± SEM (arbitrary units) for each specific mRNA quantification using 18S rRNA levels to normalize the results. Protein histograms show means ± SEM for each sample group. A representative image of β2-microglobulin immunoblot is shown (upper side). Statistical significance of differences between patients and controls is shown as *, and between subcutaneous (SAT) and visceral (VAT) controls or patients is shown as #.

Table 1. Demographics, treatment data, anthropometric and biochemical parameters in patients and controls.

	CONTROL		HIV-infected-HAART-Treated patients with lipodystrophy			p	p
Parameters	Subcutaneous (n=10)	Visceral (n=10)	Subcutaneous (n=10)	Visceral (n=8)	p	(C vs HIV+)	(SAT vs VAT)
Age (years) (± SD)	46.0 ± 5.1	57.5 ± 5.9	41.5 ± 2.6	46.4 ± 2.8	0.08	0.33	0.31
Sex (% male)	60	30	60	60	0.47	0.11	011
BMI	21.3 (2.1)	24.8 (3.3)	25.2 (7.4)	25.5 (9.7)	0.38	0.38	0.47
WHR	0.90 (0.09)	0.93 (0.13)	0.93 (0.11)	0.94 (0.04)	0.12	0.46	0.11
Facial lipoatrophy (Fontdevila categories 0-3)	-	-	2-3	2-3	-	-	-
Time since HIV infection (months) (± SD)	-	-	142 ± 26	154 ± 24	0.81	-	0.34
Cumulative time on NRTI (months)	-	-	102 (99)	66.57 (75)	0.32	-	0.32
Cumulative time on NNRTIs (months)	-	-	27 (5)	23 (13)	0.38	-	0.38
Cumulative time on PIs (months)	-	-	42.25 (34.5)	36.29 (37)	0.68	-	0.68
Triglycerides (mmol/l)	0.83(0.6)	0.91 (0.5)	2.3 (1.2)	2.26 (1.6)	0.09	**0.02**	0.31
Cholesterol (mmol/l)	5.21 (1.3)	4.80 (1.4)	4.84 (2.2)	5.16 (2.7)	0.83	0.62	0.73
Glucose (mmol/l)	4.9 (0.9)	5.0 (0.2)	5.5(0.9)	5.4 (0.5)	0.12	**0.04**	0.27
HOMA-R	0.79 (0.9)	0.85 (0.9)	2.1 (0.9)	2.2 (1.1)	**0.03**	**0.01**	0.1
CD4+ T-cell count (cells/mm3)	-	-	790 (891)	727 (897)	0.88	-	0.88
HIV-1 RNA (log10 copies/ml)	-	-	2.3 (0.9)	2.0 (2.2)	0.7	-	0.7

Unless indicated values are expressed as median (inter-quartile range). BMI body mass index; WHR waist-to-hip ratio; NRTI nucleoside reverse transcriptase inhibitors; NNRTI non-nucleoside reverse transcriptase inhibitors; PI protease inhibitors; HOMA-R homeostasis model assessment for insulin resistance; p is total p-value between groups; C means controls ; HIV+ means HIV infected patients; SAT is subcutaneous; VAT is visceral adipose tissue.


A)
mtDNA content
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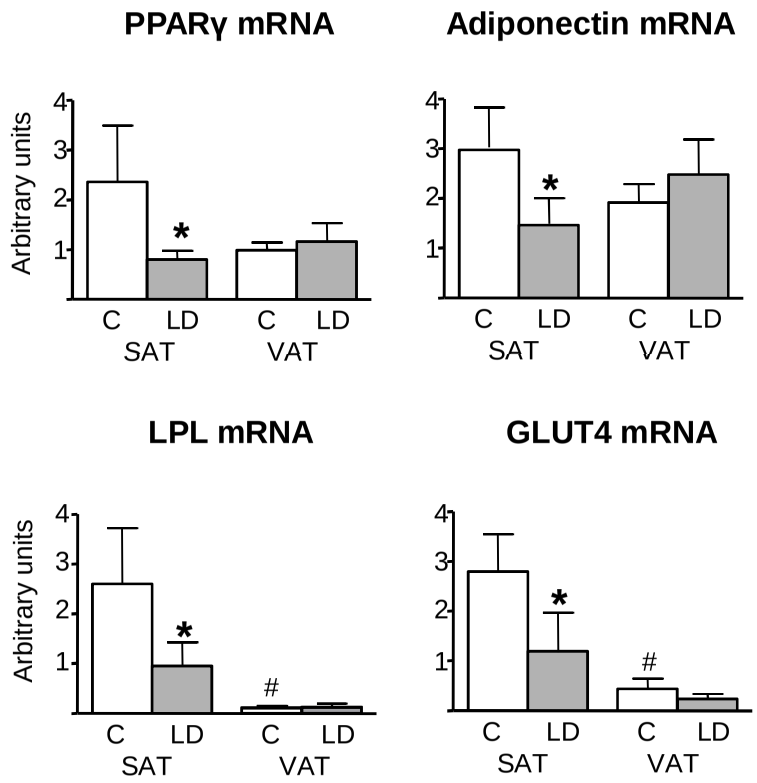
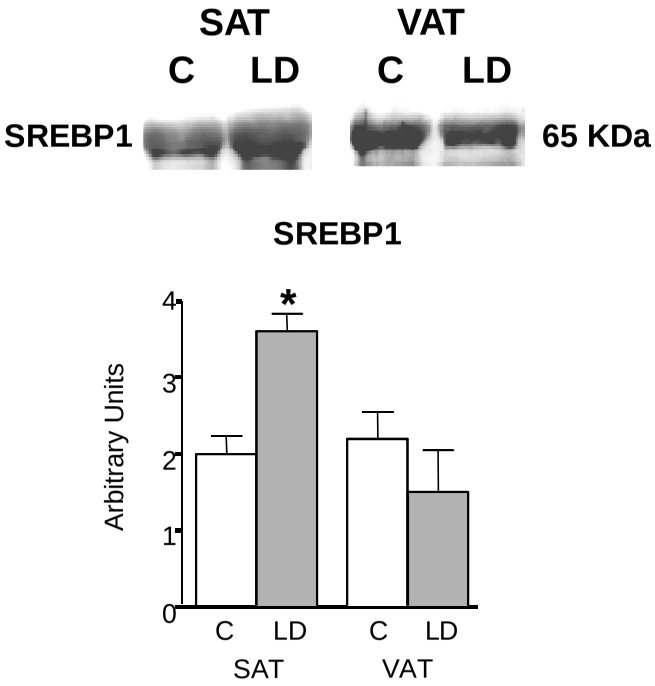
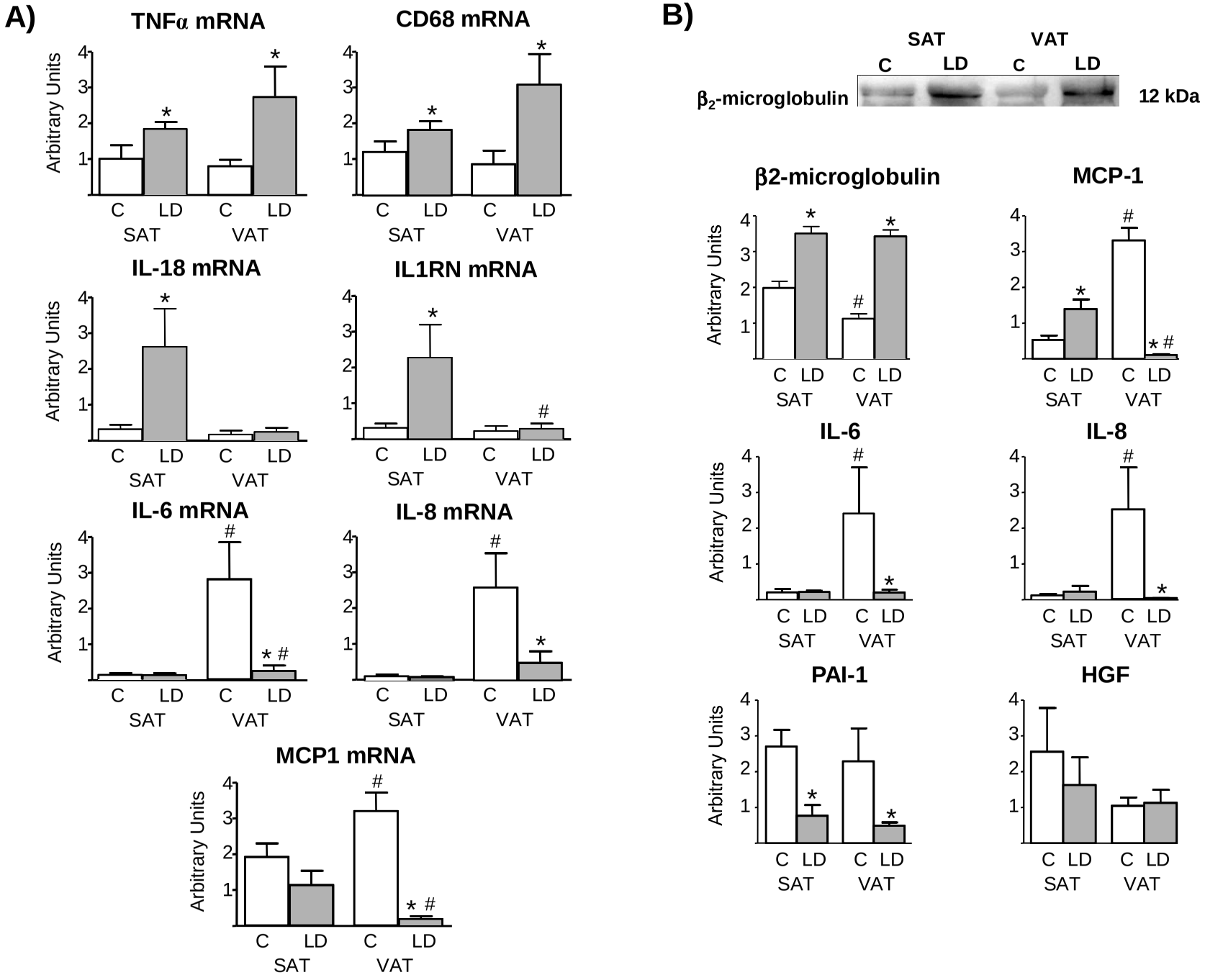
B)
Cyt b mRNA
COII mRNA
Cyt c mRNA
COIV mRNA
C)
SAT VAT
C LD C LD
ATPsyn α
FeS
COII
COIV
55 KDa
35 KDa
30 KDa
17 KDa
ATPsyn α (Complex V)
FeS 30 kd (Complex II)
COII (Complex IV)
COIV (Complex IV)

A)


PPARγ mRNA
Adiponectin mRNA
LPL mRNA
GLUT4 mRNA
Arbitrary units
C LD C LD
SAT VAT
*
#

B)


SAT VAT
C LD C LD
SREBP1
65 KDa
SREBP1
Arbitrary Units
C LD C LD
SAT VAT
*


A)
TNFα mRNA
CD68 mRNA
IL-18 mRNA
IL1RN mRNA
IL-6 mRNA
IL-8 mRNA
MCP1 mRNA
Arbitrary Units
C LD C LD
SAT VAT
B)
SAT VAT
C LD C LD
β2-microglobulin
12 kDa
β2-microglobulin
MCP-1
IL-6
IL-8
PAI-1
HGF

Differential gene expression indicates that "buffalo hump" is a distinct adipose tissue disturbance in HIV-1-associated lipodystrophy

Revista: AIDS. 2008 Mar 12; 22(5):575-84.

PMID: 18316998

Título: Una expresión diferencial de expresión génica indica que la "buffalo hump" (giba de búfalo) es una alteración diferente del tejido adiposo en la lipodistrofia asociada a la infección por HIV-1

El objetivo de este artículo era determinar las bases moleculares del crecimiento progresivo de tejido adiposo dorso-cervical que aparece en un subgrupo de pacientes infectados por el virus HIV-1 con lipodistrofia asociada a terapia HAART llamado "buffalo hump" (BH) o giba de búfalo. Para ello se llevó a cabo un análisis de la expresión de marcadores génicos de función mitocondrial, adipogénesis, inflamación y proliferación celular en 10 muestras de tejido adiposo de "buffalo hump" y 10 muestras de tejido adiposo subcutáneo de pacientes infectados por el virus HIV-1 y tratados con terapia HAART y 10 controles sanos. Los resultados muestran que las muestras de los lipomas dorso-cervicales de los pacientes infectados presentan niveles más bajos de DNA mitocondrial y transcritos codificados por DNA mitocondrial que los controles. Solo en las muestras de grasa de BH se observó expresión de UCP1. Por el contrario no había diferencias significativas en la expresión de UCP2, UCP3 o marcadores génicos de adipogénesis entre el tejido adiposo de BH y los controles. La grasa de los lipomas BH no mostraba la alta expresión de TNFα y β2-microglobulina identificada en las muestras de tejido adiposo subcutáneo lipoatrófico de los pacientes con lipodistrofia. El marcador de macrófagos CD68 también aparecía más bajo en BH que en las muestras subcutáneas lipoatróficas de los pacientes. En contraste, BH mostraba una expresión más alta del marcador de proliferación PCNA. De estos resultados se concluye que el tejido adiposo de las zonas lipomatosas dorso-cervicales presenta alteraciones específicas en la expresión génica respecto al tejido adiposo subcutáneo de pacientes lipodistróficos. Las alteraciones mitocondriales no pueden explicar el comportamiento de las BH respecto a los depósitos lipoatróficos. La ausencia de un estado de inflamación local en BH podría explicar en parte este comportamiento.

Differential gene expression indicates that 'buffalo hump' is a distinct adipose tissue disturbance in HIV-1-associated lipodystrophy

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Objective: To elucidate the molecular basis of the progressive enlargement of dorsocervical adipose tissue, the so-called 'buffalo hump', that appears in a sub-set of patients with HIV-1/HAART-associated lipodystrophy.

Design: Analysis of the expression of marker genes of mitochondrial function, adipogenesis, inflammation and cell proliferation in ten 'buffalo hump' samples and ten subcutaneous fat samples from HIV-1-infected/HAART-treated patients, and in ten healthy controls.

Methods: Quantitative real-time polymerase chain reaction analysis of mitochondrial DNA and gene transcripts, and immunoblot for specific proteins.

Results: 'Buffalo hump' patients had lower levels of mitochondrial DNA and mitochondrial DNA-encoded transcripts with respect to healthy controls. The uncoupling protein (UCP)-1 gene was expressed only in 'buffalo hump' fat. There were no significant changes in the expression of UCP2, UCP3 or of marker genes of adipogenesis in 'buffalo hump' patients relative to healthy controls. 'Buffalo hump' fat did not show the high expression of tumor necrosis factor-α and β2-microglobulin identified in lipoatrophic subcutaneous fat from patients. The expression of the macrophage marker CD68 was also lower in 'buffalo hump' than in subcutaneous fat from patients. In contrast, 'buffalo hump' showed a higher expression of the cell proliferation marker PCNA.

Conclusions: 'Buffalo hump' adipose tissue shows specific disturbances in gene expression with respect to subcutaneous fat from HIV-1-infected/HAART-treated patients. Mitochondrial alterations cannot explain the differential behavior of 'buffalo hump' with respect to adipose depots prone to lipoatrophy. The absence of a local inflammatory status in 'buffalo hump' may explain in part the differential behavior of this adipose tissue.

AIDS 2008, **22**:575–584

Keywords: adipocyte differentiation, adipokines, 'buffalo hump', HIV-1/HAART-associated lipodystrophy, inflammation, mitochondria, proliferation, subcutaneous adipose gene expression

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Received: 3 August 2007; revised: 30 November 2007; accepted: 11 December 2007.

Introduction

Disturbances in adipose tissue in HIV-1-infected patients under HAART involve a complex set of alterations. A large proportion of patients exhibiting the HAART-associated lipodystrophy syndrome show lipoatrophy in subcutaneous adipose tissue. Lipoatrophy occurs often in the face, arms, and legs. An enlargement in visceral adipose tissue, reminiscent of visceral obesity, also occurs with remarkable frequency, often in combination with peripheral lipoatrophy [1,2]. Lipomatosis is also commonly found in HIV-1-infected patients under HAART. It appears usually as an enlargement in the dorso-cervical area ('buffalo humps') [3,4], although other anatomical distributions (for example pubis, neck or trunk) have been also noted [5,6]. The frequency of 'buffalo hump' in HIV-1-infected patients has been reported to range from 2 to 13%, with a higher prevalence (6 to 13%) in those showing any other feature of the lipodystrophy syndrome [7]. The presence of lipomatosis often requires surgical removal due to discomfort, for aesthetic reasons or even because of localized pain. The prognosis after surgery is variable and recurrences are not unusual [8].

HAART-associated 'buffalo hump' is unlikely to be simply an additional variation of other enlargements of adipose tissue in lipodystrophy, as those occurring in the visceral area. Enlargement of adipose tissue in the dorso-cervical region or other sites showing lipomatosis involves subcutaneous fat, a type of adipose tissue that, in most of the patients showing lipodystrophy, is characterized by lipoatrophy. In some cases, patients display enlargement in localized subcutanous sites ('buffalo hump') but lipoatrophy in other, even surrounding, regions. Visceral fat accumulation is likely to be a distinct phenomenon, as it involves the intra-abdominal depot of adipose tissue. A recent report has shown that whereas increased visceral adipose tissue gives rise to a phenotype reminiscent of the metabolic syndrome, dorso-cervical fat accumulation is associated only with hyperinsulinemia [7]. Moreover, fat enlargements leading to 'buffalo hump' may result from alterations of the adipocyte cell at those sites. Adipocytes in 'buffalo hump' express the brown fat uncoupling protein-1 (UCP1) gene, thus suggesting disturbances in the brown-versus-white adipocyte differentiation pattern [9].

The etiopathogenesis of lipomatosis in the context of adipose tissue disturbances in HAART-treated, HIV-1-infected patients, is unknown. Some patients with inherited diseases involving mutations in the tRNA-Lys gene of mitochondrial DNA also show an enlarged, UCP1-expressing, region of adipose tissue in the dorso-cervical area. This led to the hypothesis of the involvement of mitochondrial disturbances in the origin of 'buffalo hump' in HIV-1-infected patients [10–12]. It may be hypothesized that 'buffalo hump' develops as a site-specific compensatory response of adipose tissue to metabolic dysregulation, although an alternative hypothesis could be that 'buffalo hump' is caused by enhanced local toxicity elicited by HIV-1 infection and/or HAART in this particular area. Evidence to support this latter model might include evidence of enhanced tissue pathology such as inflammation or mitochondrial toxicity. Understanding the molecular mechanisms of this dramatic modification in the behavior of the adipose depots is of interest in order to prevent and/or treat lipomatosis and to understand the overall etiopathogenesis of adipose tissue modifications associated with HIV-1/HAART. Moreover, it may provide information on the basic aspects of what determines the proliferative capacity of adipose cells in humans. For this purpose, we analyzed the profile of gene expression in 'buffalo hump' from HIV-1-infected patients on HAART, in comparison with that in subcutaneous fat from HIV-1-infected, HAART-treated patients showing lipoatrophy but not lipomatosis, and that from healthy non-HIV-1 infected, nontreated individuals.

Materials and methods

Gene expression in enlarged dorso-cervical adipose tissue ('buffalo hump') from ten HIV-1-positive patients on HAART was studied upon surgical removal. These patients displayed, along with 'buffalo hump', the clinical characteristics consistent with HAART-associated peripheral lipoatrophy (fat wasting from the face, buttocks and limbs). They were compared with ten HIV-1-positive patients also showing HAART-associated peripheral lipoatrophy, but without lipomatosis. Patients in both groups were similar with respect to mean age, male/female ratio, serum CD4+ cells/μl, serum cholesterol, triglycerides and virological control of HIV-1 infection (Table 1). Between the two patient groups, differences in the number of cumulative months of treatment with nucleoside analog reverse transcriptase inhibitors (NRTI), with nonnucleoside analog reverse transcriptase inhibitors (NNRTI) or with protease inhibitors (PI) were not statistically significant. Differences for cumulative treatment with the predominant NRTI (zidovudine, stavudine, lamivudine, didanosine, abacavir), NNRTI (efavirenz, nevirapine) and PI (indinavir, ritonavir/lopinavir, nelfinavir) drugs used throughout the antiretroviral treatment history of the patient groups were also not statistically significant (Table 1). For comparison, a third group was established with sex and age-matched non-HIV-1-infected, non-treated individuals. Biopsy samples of subcutaneous fat from patients showing lipodystrophy, but without 'buffalo hump', and from controls were taken from the abdominal area, whereas samples of 'buffalo hump' were taken from biopsies obtained at the time of surgical removal. All procedures were performed with informed consent from

Table 1. Demographics, treatment data and biochemical parameters in patients and controls.

Parameters	HIV-1-infected/HAART-treated patients			
	Control ($n=10$)	Lipoatrophy ($n=10$)	'Buffalo hump' ($n=10$)	*P*-value
Age (years)	38.0 ± 5.9	38.5 ± 2.0	44.8 ± 2.8	0.08
Sex (% male)	70	70	60	
Time since HIV-1 infection (months)	–	87 ± 17	125 ± 20	0.16
Cumulative time (months) on:				
NRTIs	–	142 ± 14	151 ± 19	0.70
Zidovudine		36 ± 14	18 ± 6	0.37
Stavudine		38 ± 7	39 ± 11	0.99
Lamivudine		32 ± 8	41 ± 13	0.25
Didanosine		29 ± 9	23 ± 12	0.70
Abacavir		5 ± 2	4 ± 2	0.73
Cumulative time (months) on:				
NNRTIs	–	27 ± 5	28 ± 10	0.92
Nevirapine		22 ± 6	19 ± 10	0.78
Efavirenz		5 ± 3	14 ± 7	0.21
Cumulative time (months) on:				
Protease inhibitors	–	45 ± 11	37 ± 10	0.59
Indivinavir		25 ± 7	14 ± 6	0.25
Ritonavir/lopinavir		5 ± 3	3 ± 2	0.59
Nelfinavir		3 ± 1	7 ± 4	0.35
Triglycerides (mmol/l)	0.8 ± 0.1	4.4 ± 0.3 $P<0.001$	3.1 ± 0.7 $P=0.004$	0.10
Cholesterol (mmol/l)	2.9 ± 0.4	4.5 ± 0.3 $P=0.005$	3.4 ± 0.5	0.07
$CD4^+$ T (cells/μl)	–	668 ± 47	602 ± 87	0.59
HIV-1 RNA (log_{10} copies/ml)	–	2.3 ± 0.4	2.2 ± 0.7	0.90

Values are expressed as means ± SEM. The *P*-value column corresponds to the comparison between the 'buffalo hump' and the lipoatrophy groups. *P*-values under every parameter are shown for comparison respect to controls when <0.05. NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitors.

participants and the study was approved by the Hospital de la Santa Creu i Sant Pau ethics committee. Tissue samples were frozen in liquid nitrogen. After homogenization in RLT (Qiagen, Hilden, Germany) buffer, DNA was isolated using a phenol/chloroform extraction methodology and RNA was isolated using a column-affinity based methodology, including on-column DNA digestion (RNeasy; Qiagen). One microgram of RNA was transcribed into cDNA using MultiScribe reverse transcriptase and random-hexamer primers (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, California, USA). For quantitative mRNA expression analysis, TaqMan reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed on the ABI PRISM 7700HT sequence detection system (Applied Biosystems). The TaqMan RT-PCR reaction was performed in a final volume of 25 μl using TaqMan Universal PCR Master Mix, No AmpErase UNG reagent and the following specific primer pair probes: 18S rRNA, Hs99999901; UCP1, Hs00222453; UCP2, Hs00163349; UCP3, Hs00243297; COX4I1, Hs00266371; PPARGC1, Hs00173304; CEBPA, Hs00269972; LPL, Hs00173425; TNF, Hs00174128; PPARG, Hs00234592; APM1(adiponectin), Hs00605917; Pref-1, Hs00171584; b2microglobulin, Hs99999907; CD68, Hs00154355.; COL1A2, Hs00164099 (TaqManGene Expression Assays; Applied Biosystems). Primers and probe for the detection of cytochrome *c* oxidase subunit II (COII) mRNA and mtDNA abundance assessment were designed (Custom TaqMan Gene Expression Assays; Applied Biosystems) and the sequences were: CAAACCACTTTCACCGCTAC AC (forward) and GGACGATGGGCATGAAACT GT (reverse) and the FAM-labeled probe was AAATCT GTGGAGCAAACC. Quantification of mtDNA was referred to nuclear DNA as determined by the amplification of the intronless gene CEBPα. Controls with no RNA, primers, or RT were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA expression of the genes of interest which were normalized to that of the reference control (18S ribosomal RNA) [13] using the comparative ($2^{-\Delta CT}$) method, following the manufacturer's instructions. Parallel calculations using the PPIA reference gene (Hs99999904) were performed and results were essentially the same.

For immunoblot quantification of protein expression, adipose tissue samples were homogenized in cold buffer (10 mmol/l HEPES pH 7.5, 5 mmol/l ethylenediamine tetra-acetic acid, 5 mmol/l dithiothreitol, 5 mmol/l $MgCl_{2)}$, and protease inhibitors (Complete-mini; Roche, Sant Cugat del Vallès, Spain). 40 μg of homogenate protein were mixed with equal volumes of 2 × sodium dodecyl sulphate (SDS) loading buffer, incubated at 90°C for 5 min, and electrophoresed on SDS/polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Millipore, Billerica, Massachusetts, USA) and immunological detection was performed using antibodies

against PPARγ, SREBP1 and PCNA (H-100, K-10 and sc-25280, respectively, Santa Cruz Biotechnology, Santa Cruz, California, USA), β2-microglobulin (P0163; Dako Cytomation, Glostrup, Denmark), VDAC (31HL-529536; Calbiochem, San Diego, California, USA), COII (12C4; Molecular Probes, Eugene, Oregon, USA) or β-actin (Sigma A5441; Sigma, St Louis, Missouri, USA). Immunoreactive complexes were detected using an antirabbit secondary antibody (sc-2004; Santa Cruz Biotechnology) and the enhanced chemiluminiscence detection system (Amersham Biosciences, Little Chalfornt, Buckinghamshire, UK). The intensity of the signals was quantified by densitometry (Phoretics 1D Softward; Phoretic International Ltd, Newcastle, UK).

Results were expressed as means ± SEM and statistical comparison for differences were performed using nonparametric analysis of variance. Statistical significance of differences was established when P values were < 0.05.

Results

Adipose tissue from 'buffalo hump' showed a significant depletion in mitochondrial DNA (mtDNA) with respect to control adipose tissue (Fig. 1), that was even more pronounced than in nonlipomatous subcutaneous fat from patients. The levels of COII mRNA, a mtDNA-encoded transcript, paralleled these findings, thus indicating an impairment in the synthesis of mtDNA-encoded transcripts as a consequence of mtDNA depletion. The expression of the mRNA for COIV, a nuclear-encoded subunit of the respiratory chain complexes, was similarly lower in 'buffalo hump' adipose tissue and in subcutaneous adipose tissue from patients.

In accordance with a previous report [9], 'buffalo hump' adipose tissue showed substantial levels of expression of UCP1 mRNA, which was not detected in subcutaneous fat from healthy controls or from patients showing only lipoatrophy. UCP2 mRNA and UCP3 mRNA levels were not significantly altered in 'buffalo hump' fat relative to controls (Fig. 1), despite the fact that these transcripts levels were significantly lower and higher, respectively, in subcutaneous fat from patients, as already reported [14].

PPARγ mRNA levels in subcutaneous adipose tissue, but not in 'buffalo hump', from patients were substantially lower than those in healthy controls. A similar tendency was observed for C/EBPα mRNA expression although the changes were not statistically significant (Fig. 2).

The expression levels of both PGC-1α mRNA and Pref-1 mRNA, which were significantly higher in subcutaneous

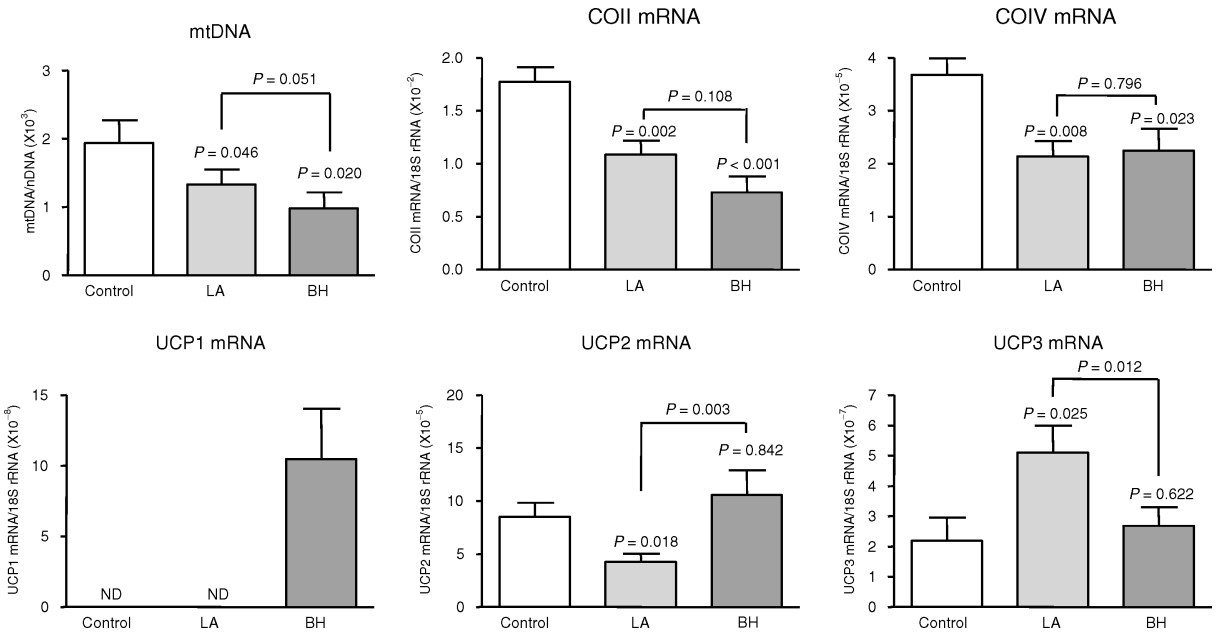

Fig. 1. mtDNA levels and mRNA levels of mitochondrial function marker genes in 'buffalo hump' (BH) and in subcutaneous adipose tissue from HIV-1-infected, HAART-treated patients, compared with subcutaneous adipose tissue from healthy controls. The figure shows means ± SEM for mtDNA concentration (expressed as a ratio relative to nuclear DNA) and for each specific mRNA concentration (expressed as ratios relative to 18S rRNA). The P values for statistical comparisons relative to the control group are shown in the top of the bars and those between the lipoatrophy (LA) and BH groups in brackets. Differences were considered to be statistically significant when $P < 0.05$. ND = not detectable.

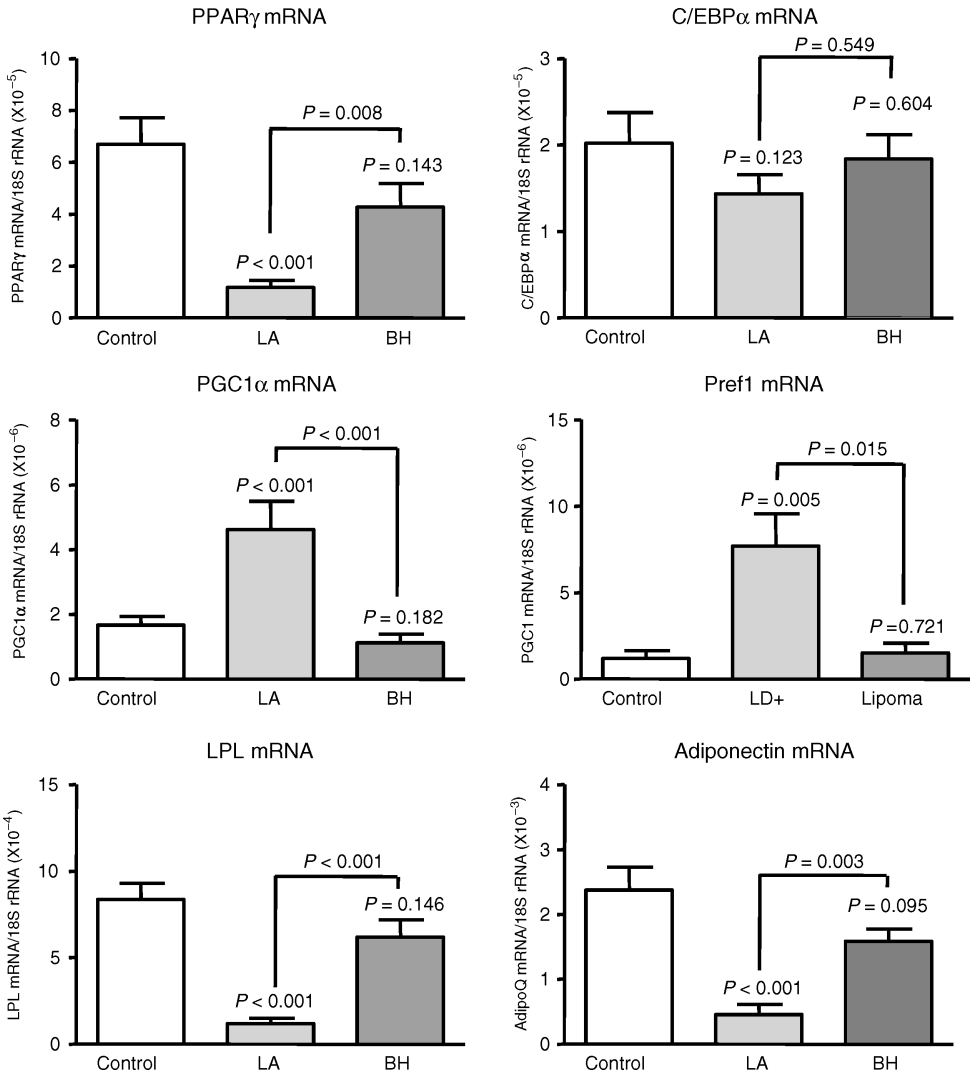

Fig. 2. mRNA concentrations of marker genes of adipogenesis in 'buffalo hump' (BH) and in subcutaneous adipose tissue from HIV-1-infected, HAART-treated patients, compared with subcutaneous adipose tissue from healthy controls. The figure shows means ± SEM for each specific mRNA concentration (expressed as ratios relative to 18S rRNA). The *P* values for statistical comparisons between groups are shown as in Fig. 1. LA = lipoatrophy.

fat from patients showing lipoatrophy, were also not significantly different in 'buffalo hump' adipose tissue relative to those in controls. Similarly, lipoprotein lipase mRNA and adiponectin mRNA levels, which were lower in subcutaneous adipose tissue from lipoatrophic patients, were not significantly different in 'buffalo hump' fat.

TNFα mRNA levels were not significantly higher in 'buffalo hump' relative to control adipose tissue (Fig. 3). This is in contrast with those in subcutaneous fat from patients, which did show significantly higher levels, as previously reported [14–16]. The mRNA expression of β2-microglobulin, a target gene of inflammatory cytokines [17,18], was also not significantly different in 'buffalo hump' with respect to control adipose tissue (Fig. 3). As a relevant component of the local inflammation status of subcutaneous fat in patients showing lipodystrophy has been attributed to enhanced macrophage infiltration and activation in adipose tissue, the transcript levels of CD68, a marker of macrophages, were determined. Subcutaneous adipose tissue, but not 'buffalo hump' fat, in patients showed moderately higher levels of CD68 mRNA with respect to control adipose tissue. In fact, CD68 mRNA levels were significantly lower in 'buffalo hump' than in subcutaneous fat from patients. Finally, considering reports of enhanced fibrosis in 'buffalo hump' [8,19], we analyzed the mRNA levels of the pro-alpha2(I) collagen (COL1A2), a marker of fibrosis [20]. Differences between groups were not statistically significant.

To further analyze the changes in gene expression in 'buffalo hump', we studied the protein levels of

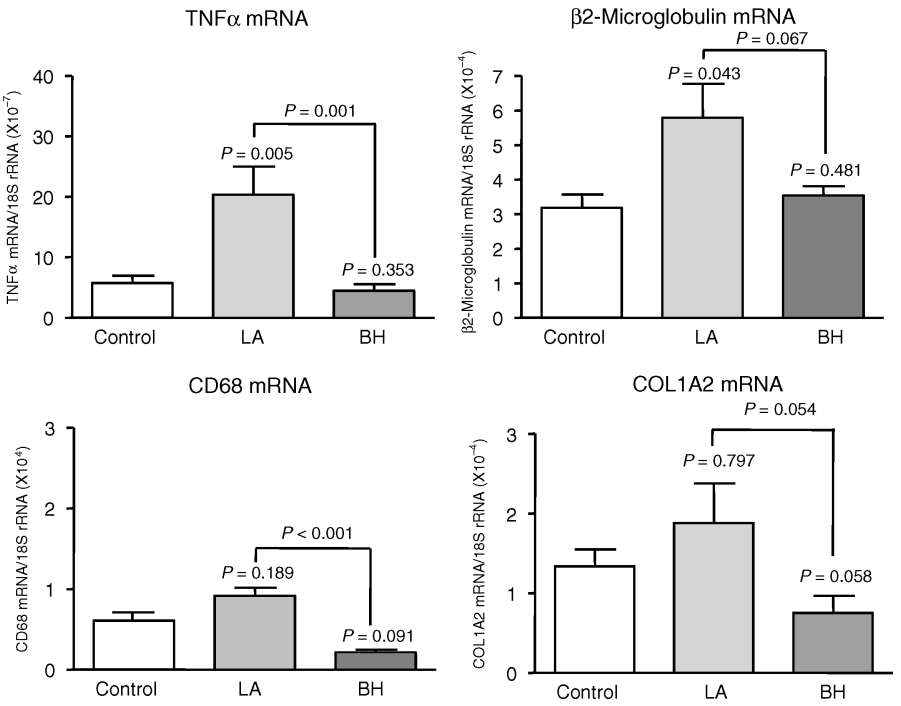

Fig. 3. mRNA concentrations of TNFα, β2-microglobulin, CD68 and COL1A2 in 'buffalo hump' (BH) and in subcutaneous adipose tissue from HIV-1-infected, HAART-treated patients, compared with subcutaneous adipose tissue from healthy controls. The figure shows means ± SEM for each specific mRNA concentration (expressed as ratios relative to 18S rRNA). The *P* values for statistical comparisons between groups are shown as in Fig. 1. LA = lipoatrophy.

representative marker genes of mitochondrial function, either mtDNA-encoded (COII) or nuclear-encoded and unrelated to the respiratory chain [voltage-dependent anion carrier (VDAC)], of adipogenesis (PPARγ) and of inflammation status (β2-microglobulin). Moreover, we also analyzed the protein levels of SREBP1, an adipogenic transcription factor which is post-transcriptionally regulated and is profoundly altered in subcutaneous adipose tissue from HIV-1-infected, HAART-treated, patients showing lipoatrophy [15]. Protein levels of 'proliferating cell nuclear antigen' (PCNA), a marker gene of cell proliferation, were also determined.

The results indicated that COII protein abundance, relative to overall mitochondrial protein, was lower in subcutaneous adipose tissue from patients. Even lower levels of COII protein were found in 'buffalo hump' fat, in parallel with data above on mtDNA and COII mRNA levels (Fig. 4). In contrast, the analysis of overall mitochondrial protein levels in patients, as estimated through VDAC protein abundance, indicated higher levels in subcutaneous fat and also, and more intensely, in 'buffalo hump'.

Changes in PPARγ protein levels paralleled those observed for the corresponding transcripts: lower levels in subcutaneous fat from patients with respect to healthy controls and no significant changes in 'buffalo hump' fat (Fig. 4). For β2-microglobulin, in 'buffalo hump' not only were there not the high levels of expression observed in subcutaneous fat from patients, but levels were even lower than in healthy controls. The expression levels of SREBP1 were dramatically higher in subcutaneous adipose tissue from patients relative to those in controls; however, no such alteration was observed in 'buffalo hump'. Concerning PCNA, 'buffalo hump' adipose tissue showed higher levels of expression with respect to subcutaneous fat from patients or from healthy controls, although the abundance of PCNA protein was remarkably variable in 'buffalo hump' samples from different patients.

Discussion

Whereas several studies have addressed the molecular basis of subcutaneous adipose tissue lipoatrophy through the analysis of gene expression in adipose tissue [14,15,21,22] no such study was available regarding 'buffalo hump' fat and only a single previous report indicated the expression of UCP1, the marker gene of brown fat [9]. The present findings confirm UCP1 gene expression as a disturbance that is highly specific to 'buffalo hump' adipose tissue, not shared by lipoatrophic subcutaneous adipose tissue from patients equally infected by HIV-1 and being under HAART. This establishes that alterations in the white-versus-brown differentiation pattern of adipocytes is a

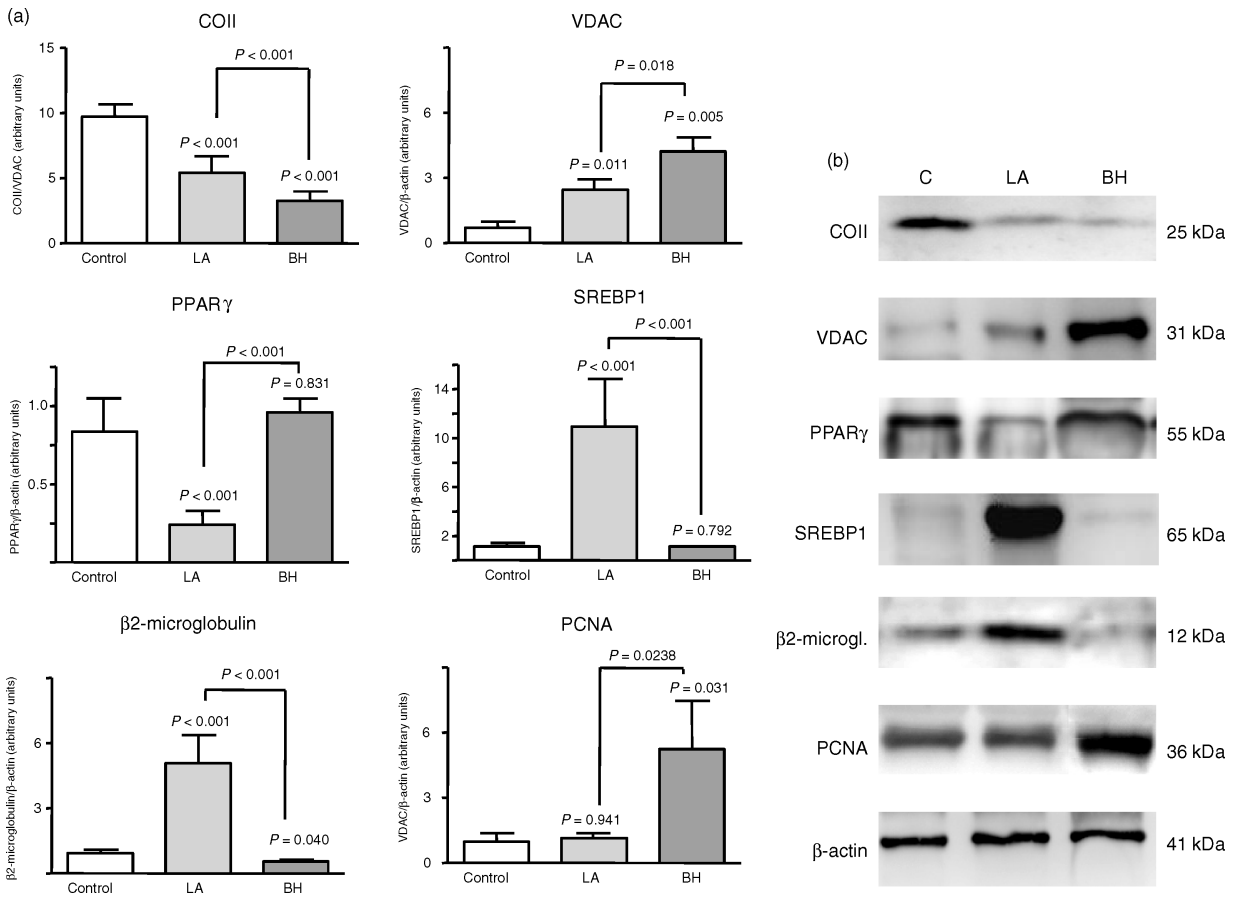

Fig. 4. Protein levels of marker genes of mitochondrial function, adipogenesis, inflammation, and cell proliferation in 'buffalo hump' (BH) and in subcutaneous adipose tissue from HIV-1-infected, HAART-treated patients, in comparison with subcutaneous adipose tissue from healthy controls. (a) Means ± SEM for the specific immunoreactive signals for the indicated proteins relative to β-actin or VDAC. The *P* values for statistical comparisons between groups are shown as in Fig. 1. (b) Examples of immunoblot assays. Each sample corresponds to an individual from each group. On the right, the molecular weight of the specific immunoreactive signal is shown. LA = lipoatrophy.

characteristic feature only of 'buffalo hump' and not a common feature of adipose tissue disturbances associated with HAART. Stavudine and nevirapine have been reported to induce UCP1 gene expression and brown adipocyte differentiation in cell culture [23]. The limited number of samples and the cross-sectional nature of the present study prevent us from establishing unequivocally the potential role of drug treatment in the differences between UCP1 gene expression in 'buffalo hump' and in subcutaneous fat. A previous report, however, indicated that the appearance of 'buffalo hump' could not be associated with any specific component of HAART regimes [7]. Another feature of 'buffalo hump' fat, enhanced mitochondrial biogenesis, is also consistent with this acquisition of a brown fat-like phenotype in this adipose depot. The expression of both PGC-1α and UCP3 is not, however, significantly different with respect to controls, in contrast to the high expression observed in lipoatrophic areas. These two genes are preferentially expressed in brown rather than white, adipose tissue [24,25], thus indicating that 'buffalo hump' acquires some features of brown adipose tissue but not a full-blown brown fat phenotype. This is in agreement with reports of a morphology intermediate between typical brown and white in adipocytes from 'buffalo hump' [26]. On the other hand, due to the high sensitivity of the UCP3 gene to nonesterified fatty acids [27], the high UCP3 mRNA expression in subcutaneous fat of patients with lipoatrophy has been attributed to enhanced lipolysis [14], a phenomenon unlikely to occur in 'buffalo hump', a fat-accumulating tissue.

The similar low levels of mtDNA and mtDNA-encoded components of the respiratory chain in 'buffalo hump' and subcutaneous fat from patients is consistent with mitochondrial toxicity being common to both tissues. This indicates that mtDNA depletion is unlikely to be a key event determining whether a given adipose depot will undergo atrophy or hypertrophy in patients under HAART. This is also consistent with the lack of significant differences in the patterns of antiretroviral treatment in patients showing 'buffalo hump' or only

lipoatrophy. Recent studies in noninfected patients indicated that it is antiretroviral treatment that causes impaired mitochondrial gene expression [28]. Moreover, 'buffalo hump' and subcutaneous fat from patients both show high levels of overall mitochondrial protein. This phenomenon has already been observed in lipoatrophic adipose tissue from patients under HAART regimes containing nucleoside analog transcriptase inhibitors [29] and has been interpreted as an attempted adaptive reaction to the altered mitochondrial function due to the low mtDNA levels in adipose tissue.

Concerning adipogenic regulators, PPARγ expression is not significantly lower in 'buffalo hump', in contrast with subcutaneous fat from patients. Conversely, the high expression of the antiadipogenic factor Pref-1 in subcutaneous fat does not occur in 'buffalo hump'. This is consistent with lack of lipoatrophy and progressive increase in adipose mass in the 'buffalo hump' depot. The expression of genes associated with fat replenishment, such as lipoprotein lipase or adiponectin, which are targets of PPARγ [30,31], is consistently higher relative to expression levels in subcutaneous fat. On the other hand, a high expression of SREBP1 protein in adipose tissue has been reported in subcutaneous fat from HIV-1-infected, HAART-treated patients, showing peripheral lipoatrophy [15] and this has been attributed to the accumulation of abnormally processed SREBP1 protein. This phenomenon is also evident in subcutaneous fat from patients in the present study but, similarly to the other adipogenic regulators, it does not occur in 'buffalo hump'.

A local inflammatory environment appears to be a prominent feature of subcutaneous fat in HIV-1-infected, HAART-treated patients, as evidenced by the high expression of TNFα and other pro-inflammatory cytokines [14–16]. Many of the gene expression disturbances in subcutaneous fat from lipoatrophic areas, such as low levels of PPARγ expression and the subsequent low expression of markers of adipogenesis can be explained by the repression exerted by the high levels of TNFα. The unaltered expression of TNFα and of β2-microglobulin, a direct target of pro-inflammatory cytokines, in 'buffalo hump' is consistent with unaltered levels of adipogenic transcription factors and their targets. Thus, although the mechanisms by which HIV-1 infection plus HAART does not led to an increased inflammatory local environment in the anatomical site corresponding to 'buffalo hump' are unknown, the present observation provides an explanation for the lack of impairment in adipogenesis and why there is no lipoatrophic behavior in the 'buffalo hump' fat pad. Microscopic analysis of adipose tissue from lipoatrophic sites in patients indicate increased infiltration of macrophages which can contribute to the local inflammation [32–34]. Such induction could be observed to some extent in the subcutaneous fat from patients through the analysis of gene expression of the macrophage marker CD68 [35]. Remarkably, CD68 mRNA expression was significantly lower in 'buffalo hump' thus indicating that macrophage infiltration is not enhanced.

The high expression of PCNA supports the notion of a transformed phenotype in adipose cells in 'buffalo hump' indicating an intrinsic enhancement in cell proliferation. Auto-transplantation of adipose tissue from 'buffalo humps' to facial lipoatrophic areas in reconstructive surgery may result in an enlargement of adipose tissue cheeks, a former lipoatrophic area [36]. This indicates that cells in the 'buffalo hump' may have acquired a high proliferative capacity that remains even when they are placed in a lipoatrophic environment. PCNA expression was remarkably variable among different 'buffalo hump' samples, however, thus suggesting a distinct extent of acquisition of the proliferative status. This may explain why adipose tissue cheek enlargement after auto-transplantation occurs only in a sub-set of patients. Finally, we did not find statistically significant differences in the expression of the COL1A2 gene, a marker of enhanced fibrosis. This indicates that such phenomenon, if present in the 'buffalo hump' samples analyzed here, does not result in a massive change in gene expression.

Several limitations affect the extent of the conclusions that can be drawn from the present study. The low number of samples limits the capacity to unequivocally clarify issues such as the role of gender, treatment drug patterns or the actual significance of gene expression data when differences between groups lack statistical significance. Moreover, the lack of availability of biopsies from 'buffalo hump' and subcutaneous adipose tissue from the same individuals also precludes a proper assessment of the impact of individual variability on gene expression patterns in adipose tissue.

Despite these limitations, the present results constitute the first systematic analysis of gene expression in 'buffalo hump' adipose tissue from HIV-1-infected patients on HAART and reveal a markedly differential pattern of alterations in gene expression with respect to subcutaneous fat of patients showing lipoatrophy. Whereas low mtDNA abundance and expression is common to the two adipose tissue types, 'buffalo hump' adipose tissue shows unaltered expression of marker genes of adipogenesis, and altered gene expression indicating enhanced cell proliferation and features of a brown-versus-white fat phenotype. The lack of signs of local inflammation in 'buffalo hump' may suggest that enhanced expression of pro-inflammatory cytokines is an important determinant of the specific lipoatrophic behavior of several subcutaneous adipose tissue areas and is consistent with the hypothesis that 'buffalo hump' may represent a dysregulation of adipose tissue in this area, rather than being a reflection of enhanced toxicity. It may also be the case, however, that enhanced inflammation is a response to adipocyte death in lipoatrophic areas and not a cause of

the pathology. As with all cross-sectional analyses, this study was not designed to determine cause-and-effect relationships, and unmeasured bias inherent to this type of study may influence the observed changes. Therefore, further studies would be required to establish unequivocally whether a lack of inflammatory signals is a cause or consequence of the lipoatrophic versus proliferative status of adipose depots in patients. It has, however, been reported that adipocytes from 'buffalo hump' release much less TNFα than those from abdominal subcutaneous adipose tissue from HIV-1-infected, HAART-treated, patients when incubated 'in vitro' [37].

In summary, 'buffalo hump' is a highly specific alteration in the context of adipose disturbances in HIV-1-infected, HAART-treated patients, and distinct molecular events are associated with their induction: acquisition of brown-fat-like features, induction of proliferation but unaltered inflammatory status and adipogenesis. Furthermore, present results suggest that treatment of HAART-associated lipodystrophy in HIV-1-infected patients that is focused on promoting adipose cell differentiation or proliferation may carry a risk of enhancing lipomatosis.

Acknowledgements

Sponsorship: This study was supported by the Ministerio de Educación y Ciencia (SAF2005-01722), Fundación para la Investigación de la SIDA en España (FIPSE) (36752/06, 36610/06) and Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo (FIS-PI052336) and Red de Investigación en SIDA (RD06/006/0022), Spain.

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Hypertrophied facial fat in an HIV-1-infected patient after autologous transplantation from "buffalo hump" retains a partial brown fat-like molecular signature

Revista: Antiviral Therapy (enviado)

Titulo: La grasa facial hipertrofiada de un paciente infectado por el virus HIV-1 obtenida por trasplante autólogo de una "buffalo hump" retiene características moleculares de un tejido adiposo de tipo parcialmente marrón

La lipoatrofia facial es una alteración común entre los pacientes infectados por el virus HIV-1 tratados con antirretrovirales y a menudo suele ser corregida usando autotrasplantes. En algunos casos, cuando el paciente sufre lipomatosis en la zona dorso-cervical ("buffalo hump") y esta grasa se usa para el trasplante, la grasa trasplantada desarrolla una progresiva hipertrofia en la cara. Para determinar la base molecular de este fenómeno evaluamos la morfología celular y las características de expresión genética de esta grasa hipertrofiada. La grasa facial aumentada no mostraba una morfología reconocible como tejido adiposo marrón, pero mostraba un tamaño de adipocitos muy heterogéneo, como en las "buffalo hump". La grasa facial hipertrofiada mantuvo las características moleculares de un tejido adiposo con un fenotipo de tipo parcialmente marrón, incluyendo la expresión de UCP1, así como características adipogénicas e inflamatorias típicas de "buffalo hump". Por tanto podemos concluir que a pesar de ser implantado en una zona facial lipoatrófica, la grasa obtenida de una "buffalo hump" mantiene su fenotipo alterado y su capacidad para hipertrofiarse progresivamente. Estos resultados indican que los depósitos lipomatosos dorsocervicales deben ser considerados con precaución como fuente para trasplantes autólogos en pacientes de este tipo.

Hypertrophied facial fat in an HIV-1-infected patient after autologous transplantation from "buffalo hump" retains a partial brown fat-like molecular signature

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Word count: 1643

Running title: Hypertrophied facial fat molecular signature

Sources of support: Ministerio de Ciencia e Innovación (grant SAF2011-23636), Instituto de Salud Carlos III (grant PI11/00376) and Red de Investigación en SIDA, Spain.

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Background. Facial lipoatrophy, a common alteration among HIV-1-infected, antiretroviral-treated patients, is often corrected using autologous transplantation. In some cases, especially when enlarged adipose tissue from the dorso-cervical area ("buffalo hump") is used as a source of fat for transplantation, progressive hypertrophy of the transplanted fat develops in the face. To gain insight into the molecular basis of this phenomenon, we evaluated the cell morphology and gene expression signature of this hypertrophied facial fat.

Methods. The gene expression pattern in a sample of facial hypertrophied fat that developed after autologous transplantation was analyzed using quantitative real-time polymerase chain reaction and compared with that in "buffalo hump" and in subcutaneous fat from healthy controls. Optic and electron microscopic analyses were also performed to determine cell morphology.

Results. Enlarged facial adipose tissue did not exhibit overt microscopic morphology of brown adipose tissue although, like "buffalo hump" fat, contained adipocytes highly heterogeneous in size. Enlarged facial fat retained the molecular signature of a distorted brown-to-white adipocyte phenotype, including expression of uncoupling protein-1 (UCP1), as well as unaltered adipogenesis and inflammation characteristic of "buffalo hump".

Conclusions. Despite being implanted in a former lipoatrophic area, facially grafted "buffalo hump" tissue appears to retain the altered phenotype of dorso-cervical adipose cells, thus accounting for its progressive enlargement. These results argue that "buffalo hump" fat depots should be considered with caution as a source of fat for autologous transplantation.

Key words: lipodystrophy, transplantation, adipose tissue, lipomatosis

Introduction

Autologous transplantation is used commonly to correct facial lipoatrophy in HIV-infected, antiretroviral-treated patients with lipodystrophy [1]. Enlarged dorso-cervical adipose tissue ("buffalo hump") is sometimes used as source of fat tissue for transplantation. A characteristic of cells in the "buffalo hump" adipose depot is an abnormally high expression of the UCP1 gene, the molecular marker of brown adipose tissue, in addition to unaltered expression of pro-inflammatory and adipogenic genes, and enhanced cell proliferation [2,3]. Additional studies have also indicated enhanced expression of 5'-deiodinase, another marker of brown adipose tissue, in "buffalo hump" fat [4]. In some cases, autologous transplantation of "buffalo hump" adipose into the face leads to a progressive enlargement of the transplanted adipose fat; this facial lipohypertrophy, also referred to as "hamster syndrome", requires subsequent surgical intervention to remove the enlarged tissue [5]. Here we report the case of a patient experiencing facial lipohypertrophy after autologous transplantation of dorso-cervical adipose tissue that required surgical removal. We performed a gene expression analysis of a sample of this facial hypertrophied adipose mass, providing the first characterization of the molecular signature of adipose tissue that becomes enlarged following implantation into the face, an otherwise lipoatrophy-prone area

Methods

The patient was a HIV-1-infected 42-year-old woman with an 8-year history of antiretroviral treatment, who began showing characteristic features of facial lipoatrophy after 5 years of treatment. During surgery to remove "buffalo hump" fat, she underwent autologous transplantation of removed dorso-cerival adipose tissue into the cheek. One year later, she developed a progressive enlargement of cheek adipose tissue that required surgical removal. A sample of the extracted adipose tissue was used for gene expression and microscopy analyses. Microscopic morphology and transcript levels of marker genes in the patient's sample were compared with reference microscopy images and with mean values for transcript levels in "buffalo hump" from HIV-1-infected, antiretroviral-treated patients, and subcutaneous fat from healthy controls obtained in the upper trunk dorsal area on occasion of minor dermatological surgery. The demographics of the reference patient groups (10 individuals/group) and viral infection-related data and treatment information have been previously reported [2]. Age, mean time since HIV-1 infection, and exposure to major antiretroviral drug families for our patient were within the range of the interquartil variability of the two reference groups.

For microscopic morphology analysis, samples were post-fixed in 1% osmium tetroxide and 0.8% FeCNK in 0.1 M phosphate buffer, dehydrated and embedded in Spurr resin. Ultrathin sections were obtained (Ultracut-UCT, Leica, Germany).White adipocyte areas were determined using the ImageJ analysis software. At least eight independent images from the facial adipose tissue samples and eight images from three distinct individual samples of "buffalo hump" and control adipose tissue were

analyzed. Statistical significance was determined by analysis of variance (ANOVA) for differences among means (± SEMs) and by Fisher analysis of variance for differences among variances. Tissue samples were also stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (JEOL-1010, Japan).

RNA was isolated (RNeasy, Qiagen, Hilden, Germany). TaqMan reverse transcription (RT) and polymerase chain reaction (PCR) reagents (Applied Biosystems, USA) were used for mRNA analysis. One microgram of RNA was transcribed into cDNA using random-hexamer primers and RT-PCR was performed (ABI PRISM 7700HT). TaqMan RT-PCR reaction were performed using TaqMan Universal PCR Master Mix, NoAmpErase UNG reagent, and TaqMan-supplied specific gene transcripts primers and probes. Controls with no RNA, primers, or reverse transcriptase were performed. Samples were tested in duplicate, and the amount of mRNA for the gene of interest was normalized to that of the reference control (18S rRNA) using the comparative (2-ΔCT) method. The results from the hypertrophied facial adipose tissue were considered different when differed from the 95% confidence interval of the reference values of "buffalo hump" fat or subcutaneous adipose tissue from healthy controls, as in previous studies of individual samples from rare forms of adipose tissue alterations [6,7].

Results

Microscopic evaluations of the hypertrophied facial adipose tissue revealed a cellular morphology typical of white adipose tissue (Fig 1). No presence of cells with obvious brown adipocyte-like morphology (multilocular fat droplets, mitochondria enrichment) were found. Similar results were obtained in samples from "buffalo hump" adipose tissue and, as expected, in control subcutaneous adipose tissue. Further examination using low-grade transmission electron microscopy confirmed the absence of typical brown adipocytes in facial enlarged adipose tissue and in "buffalo hump" (not shown). A quantitative analysis of cell surface area revealed that the mean surface of adipocytes in the hypertrophied facial adipose tissue was similar to that in "buffalo hump" samples and control fat (Table 1). However, both hypertrophied facial fat and "buffalo hump" tissue showed markedly and significantly higher variance of adipocyte size relative to that in control samples. This indicates that adipocytes in "buffalo hump" are more heterogeneous in size relative to those in control subcutaneous adipose tissue, and this alteration was retained in the hypertrophied, facially engrafted adipose tissue resulting from transplantation of "buffalo hump" fat.

Expression of marker genes in the hypertrophied facial adipose tissue sample in comparison with mean reference values in "buffalo hump" fat and subcutaneous fat from healthy controls (Table 2) indicated that autologously transplanted adipose tissue in the cheek area retained the characteristic molecular features of adipose tissue from the "buffalo hump" depot. The transcript for UCP1, an unequivocal marker of brown adipose tissue [8], was significantly expressed, at a level similar to that in "buffalo hump", in contrast with healthy control fat. Other markers of brown-versus-white adipocyte

phenotype such as SIRT3 [9], followed the same brown adipocyte-like pattern. However, as previously reported for "buffalo hump" [2], some genes considered also to be related to brown fat, such as PPARγ co-activator-α (PGC1α) and 5'-deiodinase, were unaltered in the hypertrophied facial adipose tissue sample compared to control adipose tissue. The same was observed for PRDM16, a recently identified marker gene of the brown adipocyte cell lineage [10]. This is consistent with the concept that "buffalo hump" is not composed of functional brown adipocytes, but it contains distorted adipose cells that have acquired abnormal features of brown fat. As also observed in "buffalo hump" fat, genes associated with overall adipogenic differentiation, such as PPARγ, adiponectin and lipoprotein lipase, which are down-regulated in lipoatrophic peripheral subcutaneous fat [11], were not down-regulated in the hypertrophied facial fat; in fact lipoprotein lipase transcript levels were increased. Marker genes of inflammation were not induced in the hypertrophied fat sample, and actually tended to decrease. Transcript levels for the mitochondrial DNA-encoded cytochrome c oxidase II were unchanged compared to "buffalo hump" fat but were reduced relative to healthy controls. No changes were found in transcript levels of collagen 1A2, an indicator of fibrosis.

Discussion

Our results indicate that the alterations in adipose cells caused by the combination of HIV-1 infection and antiretroviral treatment that lead to "buffalo hump" remain even after these fat cells are placed in a distinct, potentially lipoatrophic, anatomic site. In other words, adipocytes from "buffalo hump" have been permanently altered, remain insensitive to lipoatrophic stimuli occurring in the facial lipoatrophic area and retain an abnormal brown fat-like phenotype. The lack of impairment in adipogenic marker genes is also supportive of the maintenance of the characteristic "buffalo hump" gene expression signature despite placement of adipocytes in a lipoatrophic area. Regardless of the nature of the abnormal signals elicited by HIV-1 infection and antiretroviral treatment that act on dorso-cervical adipose tissue, permanent abnormal activation of the brown adipocyte lineage could be a main event in the appearance of facial lipohypertrophy after transplantation. The known high proliferative capacity of brown adipose tissue [12] would also be consistent with enlargement when pathogenic, abnormal, activation is triggered. The recent recognition of active brown adipose depots in the cervical area in adult humans [13] is consistent with this scenario, although it should be noted that, in the absence of massive enlargement, dorso-cervical adipose tissue in HIV-1-infected, antiretroviral-treated patients does not appear to express UCP1 [14]. Moreover, it has been recently shown that, notwithstanding the abnormal appearance of a brown fat-like molecular signature, "buffalo hump" does not behave as a thermogenically active brown fat [4].

This first report analyzing the features of enlarged facial fat after autologous transplantation has the obvious limitation of the population size (a single patient) and small amount of sample, which only allowed for microscopy morphology and gene expression analyses. This limitation reflects the difficulty

of accessing samples from facial fat in this type of pathology. Not all autologous transplantations of fat from "buffalo hump" result in facial lipohypertrophy; why grafted adipose tissue remains enlarging in some patients but not others is unknown. Given the variability in gene expression in "buffalo hump" pads in distinct studies [2,3,4], it is possible that only those patients in whom dorso-cervical fat pads have acquired the most intense brown fat-like, highly proliferative phenotype, risk facial lipohypertrophy following autologous transplantation. Assessment of the expression of marker genes of the brown adipocyte phenotype (e.g., UCP1) in a patient's dorso-cervical fat may be useful before using this depot as a source for autologous transplantation to correct facial lipoatrophy.

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Figures Legends

Fig 1. Example of optical microscopy analysis of adipose tissue from facial hypertrophied fat and “buffalo hump”

Table 1. Adypocyte area of hypertrofied facial fat and "buffalo hump" from HIV-1-infected patients and subcutaneous fat from healthy controls.

	Mean ± SEM ($\mu m^2 \times 10^3$)	P value for Mean ± SEM comparison	P value for variance comparison
Healthy control	53.4 ± 5.0		
Hiv-infected antiretroviral-treated			
"Buffalo hump"	46.7 ± 6.9	0.48 vs C	0.009 vs C
Hypertrophied facial adipose tissue	52.0 ± 7.3	0.88 vs C; 0.60 vs BH	0.010 vs C; 0.989 vs BH

Data are mean ± SEM of 8 independent slides from the facial fat sample, and 8 independent slides from three-different individuals from "buffalo hump" and control samples. C, healthy controls; BH, "buffalo hump".

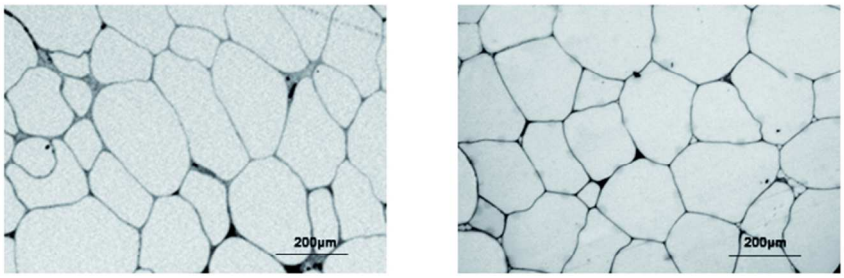

Fig 1. Example of optical microscopy analysis of adipose tissue from facial hypertrophied fat and "buffalo hump"
66x36mm (300 x 300 DPI)

Table 2. Gene expression in facial enlarged adipose tissue resulting from autologous transplantations of "buffalo hump" from HIV-1-infected patient.

	HIV-1-infected, antiretroviral-treated		Healthy controls
	Hipertrophied facial adipose tissue ("hamster syndrome")	"Buffalo hump"	Subcutaneous fat
Adipogenesis			
PPARγ	6.7	4.3 (2.2 – 6.3)	6.7 (4.4 – 9.0)
Lipoprotein Lipase	13.8 *#	6.9 (5.3 – 8.5)	8.4 (6.2 – 10.4)
Adiponectin	2.0	1.6 (1.0 – 2.1)	2.4 (1.5 – 3.2)
Inflammation			
TNFα	1.5 #	4.4 (1.4 – 6.8)	5.7 (2.0 – 8.4)
CD68	1.8 #	3.8 (0.7 – 14.4)	4.0 (2.4 – 5.9)
Mitochondriogenesis			
Cytochrome c oxidase II	0.9#	0.7 (0.4 – 1.1)	1.8 (1.3 – 2.3)
Brown-to-white adipogenesis			
Uncoupling Protein 1 (UCP1)	9.6 #	10.5 (2.3 – 18.6)	1.1 (0.1 – 2.1)
SIRT 3	3.0 #	2.6 (1.2 – 3.9)	1.9 (1.5 – 2.3)
PGC1α	1.1	2.5 (1.1 – 3.7)	1.1 (0.6 – 1.6)
5´-deiodinase (DIO2)	1.5	1.7 (0.4 – 3.0)	3.9 (1.5 – 6.4)
PRDM16	10.8	8.7 (0.6 – 16.9)	8.9 (7.1 – 10.7)
Fibrosis			
Collagen 1A2	1.9	1.8 (0.5 – 4.0)	5.0 (0.1 – 10.2)

Values correspond to mRNA levels expressed as arbitrary units for the ratio of relative abundance of the mRNA of the gene of interest relative to 18S rRNA. For the "buffalo hump" and healthy controls (10 inviduals/group) data show the mean and 95% confidence interval (in parentheses). Increased or decreased levels of mRNA expression in the facial adipose tissue hypertrophied sample respect to the confidence intervals for reference values in "buffalo hump" and healthy control samples are shown as * and #, respectively.

Reduced levels of serum FGF19 and impaired expression of receptors for hormonals FGFs in adipose tissue from HIV-1-infected patients.

Revista: JAIDS, Journal of Acquired Immune Deficiency Syndromes (ACEPTADO, Agosto 2012)

Título: Niveles en suero de FGF19 reducidos y expresión reprimida de receptores de FGF hormonales en tejido adiposo de pacientes infectados por el virus HIV-1.

El objetivo de este estudio era determinar el papel de FGF19 y los receptores de FGFs en las alteraciones metabólicas que se manifiestan en los pacientes infectados por el virus HIV-1 que siguen un tratamiento HAART. Para ello se determinaron los niveles de FGF19 en suero de 4 grupos de individuos: (1) pacientes infectados por HIV-1 en terapia HAART y que sufren lipodistrofia, (2) pacientes infectados por HIV-1 en terapia HAART y que no sufren lipodistrofia, (3) pacientes infectados por el HIV-1 no tratados y (4) individuos sanos control.

Los niveles de FGF19 en suero se correlacionaron con parámetros antropométricos, metabólicos y otros parámetros relacionados con la infección por el virus HIV-1 y la terapia HAART. Del mismo modo, se analizó la expresión génica del receptor FGFR1 y el co-receptor β-Klotho en el tejido adiposo de 10 individuos de cada uno de los grupos. Los resultados obtenidos indican que los niveles en suero de FGF19 están significativamente reducidos en los pacientes infectados por el virus HIV-1. Así, estos niveles de FGF19 en suero se correlacionan negativamente con la resistencia a insulina y niveles de insulina en sí y positivamente con el colesterol HDL. De la misma manera, los niveles de FGF19 en suero correlacionan inversamente con la exposición acumulada a los fármacos NRTI y otros no NRTI. Por otro lado se observó un claro descenso en la expresión de FGFR1 y especialmente la del co-receptor β-Klotho en todos los grupos de pacientes infectados por HIV-1.

Como conclusión cabe destacar que los niveles de FGF19 están reducidos en los pacientes infectados por HIV-1 respecto a los controles sanos. La reducción en la expresión del receptor y co-receptor que median las acciones de los FGFs hormonales en el tejido adiposo sugieren que podría existir una resistencia a los efectos metabólicos de FGF19 y FGF21 en los pacientes infectados por HIV-1. Considerando los efectos beneficiales que se han descrito recientemente de los FGFs endocrinos en el metabolismo, la reducción observada en los niveles en suero de FGF19 y una sensibilidad disminuida a los FGFs hormonales en el tejido adiposo pueden contribuir a las alteraciones metabólicas que sufren los pacientes infectados por el virus HIV-1.

Reduced levels of serum FGF19 and impaired expression of receptors for endocrine FGFs in adipose tissue from HIV-infected patients

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Key words: HIV-1, lipodystrophy, fibroblast growth factors, antiretroviral,

Running title: FGF19 and endocrine FGF receptors in HIV-1 infected patients

Conflict of interest: All authors declare no conflicts of interest

Sources of support: the study was supported by SAF2011-23636 from Ministerio de Ciencia e Innovación, grant PI11/00376 and PI10/2635 from the Instituto de Salud Carlos III; Ministerio de Sanidad Política Social e Igualdad (EC11-293); Red de Investigación en SIDA, Instituto de Salud Carlos III (RD06/0006/022 and RD06/0006/1004); and Programa de Suport a Grups de Recerca AGAUR-Generalitat de Catalunya (2009 SGR1061 and 2009 SGR284), Spain. Francesc Vidal is funded by the Programa de Intensificación de la Actividad Investigadora (ISCIII, I3SNS, INT11/240).

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Background. To determine the role of fibroblast growth factor (FGF)-19, FGF21 and the endocrine FGFs receptor system in the metabolic alterations that manifest in HIV-1-infected patients undergoing highly active antiretroviral treatment (HAART).

Methods. Serum FGF19 and FGF21 levels were determined in four groups of individuals: (1) HIV-1-infected, HAART patients with lipodystrophy (n=38), or (2) without lipodystrophy (n=34), (3) untreated ("naïve") HIV-1-infected patients (n=34), and (4) healthy controls (n=31). Serum FGF19 levels were correlated with anthropometric, metabolic, HIV-1 infection-related and HAART-related parameters and with FGF21 levels. The gene expression of FGF receptor-1 and the co-receptor β-Klotho was analyzed in adipose tissue from ten individuals from each group.

Results. Serum FGF19 levels were significantly reduced in all groups of HIV-1-infected patients, whereas FGF21 levels were increased. FGF19 levels were negatively correlated with insulin resistance and insulin levels and positively correlated with HDL-cholesterol. FGF19 was inversely correlated with cumulative exposure to nucleoside-reverse-transcriptase inhibitors (NRTI) and non-NRTI drugs. The expression of FGF receptor-1 and co-receptor β-Klotho was reduced in adipose tissue from all groups of HIV-infected patients.

Conclusions. FGF19 levels are reduced in HIV-1-infected patients, in contrast with FGF21 levels. Impaired expression of the corresponding receptor and co-receptor, which mediate the actions of endocrine FGFs in adipose tissue, suggests a resistance to the metabolic effects of FGF19 and FGF21 in HIV-1-infected patients. Considering the beneficial effects of endocrine FGFs on metabolism, the observed reduction in FGF19 levels and decreased sensitivity to endocrine FGFs in adipose tissue may contribute to metabolic alterations in HIV-1-infected patients.

INTRODUCTION

Metabolic alterations such as dyslipidemia, impaired insulin sensitivity and, in some cases, overt lipodystrophy with abnormal distribution of body fat are common in HIV-1-infected patients undergoing highly active antiretroviral treatment (HAART)[1]. Despite the presence of overt alterations is magnified in HIV-1-infected patients undergoing HAART, initial signs of altered metabolism, such as mild dyslipidemia or evidence of local inflammation in adipose tissue, have been reported in HIV-1-infected patients before starting HAART[2]. The relative involvement of HIV-1-infection-related events and HAART-specific patterns, as well as the molecular mechanisms ultimately responsible for these metabolic alterations are poorly understood.

Fibroblast growth factors (FGFs) constitute a family of regulatory proteins, most of which act as autocrine/paracrine factors and cause their biological effects in the same cells that secrete them or in closely surrounding cells. However, members of a sub-family of FGF proteins, composed of FGF19, FGF21 and FGF23 -endocrine FGFs- are known to function as hormonal regulators, acting at a distance from secreting cells[3]. FGF23 is produced by bone and regulates phosphate and vitamin D homeostasis, whereas FGF19 and FGF21 are involved in multiple aspects of metabolic regulation. FGF21 is produced mainly by the liver, and has been shown to correct glucose intolerance and hyperlipidemia in rodent models of obesity and type II diabetes[4]. FGF19 is secreted by the small intestine -mainly the ileum region- and acts on the liver to control the biosynthesis of bile acids. In addition to these effects, experimental data in rodents indicate that FGF19 has important effects that favor a healthy metabolic profile, enhancing energy expenditure in general and specifically promoting glucose disposal [5]. Some studies have reported that FGF19 impairs insulin-induced fatty acid synthesis in the liver[6] whereas other data suggested that most of the effects of FGF19 on metabolism occur through tissues other than liver, probably adipose tissue[7]. Recently, FGF19 has been reported to act as a postpandrial, insulin-independent activator of hepatic protein and glycogen synthesis, being able to restore the loss of hepatic glycogen in diabetic animals lacking insulin[8]. The metabolic effects of FGF19 and FGF21 are mediated through FGF receptors (FGFRs), predominantly FGFR4, which is expressed in liver, and FGFR1, which is mostly expressed in white adipose tissue [9,10]. Both receptors must interact with the membrane protein β-Klotho to form an FGF19/21-responsive receptor complex. Thus, β-Klotho appears to be an obligatory co-receptor for FGF19 and FGF21 responsiveness [3;9].

In a recent study we reported that the levels of FGF21 in serum were abnormally elevated in HIV-1-infected patients, especially those that developed lipodystrophy and metabolic syndrome after HAART[11]. This paradoxical observation is similar to findings in obese/diabetic patients, in which FGF21 levels are also increased [12;13]. It has been proposed that obesity constitute a

"FGF21-resistant" state[14]; and this may also occur in HIV-1-infected patients with lipodystrophy. However, it is not known whether FGF19 is altered in HIV-1 infected patients in association with their metabolic disturbances, and, hence, whether the FGF19 may be considered a potential target of treatment strategies for the metabolic syndrome in these patients, is not known. In the present study we compared FGF19 levels in HIV-1-infected patients under distinct conditions of HAART treatment and lipodystrophy with those in healthy individuals, and made a parallel assessment of FGF21 levels. We also determined the levels of FGFR1 and β-Klotho co-receptor gene expression in patient adipose tissue biopsies, to approach indications of tissue sensitivity to endocrine FGFs in HIV-1-infected patients.

METHODS

Study population

All patients and controls provided informed written consent to participate in the study. The study was approved by the ethics committee of Hospital de la Santa Creu i Sant Pau, Barcelona. Patients with oportunistic infections, neoplasms, or fever of undetermined origin were excluded from the study. At the time of the study entry, no patient or control used any other drug known to influence glucose metabolism or fat distribution, such as anabolic hormones or systemic corticosteroids, uridine, recombinant human growth hormone or appetite stimulants or suppressors. Patients with serologic evidence of infection by hepatitis viruses (B and C) were excluded from the study. For hepatitis B virus (HVB) infection exclusion, a negative serum HBV DNA was also required. No control or patient had undergone previous cholecystectomy. Patient demographics, anthropometric and metabolic data, HIV-1-infection parameters and antiretroviral treatment data are shown in Table 1. Body mass index (BMI) was calculated, and waist circumference was measured to the nearest millimeter using anatomical landmarks, as defined by the Third National Health and Nutrition Evaluation Survey [15]. Whole-body dual-energy X-ray absorptiometry (DEXA) scans (Hologic QDR-4500A Hologic, INc, 590 Lincoln St, Waltham, MA 02154, USA) were conducted by a single operator. The percentage of fat in the arms, legs and central abdomen (calculated from the mass of fat versus lean and bone mass) as well as total lean body mass (in kilograms) was recorded.

Lipodystrophy was assessed using a lipodystrophy severity grading scale (LSGS) based on that reported by Lichtenstein et al.[16] and a clinical diagnosis of lipodystrophy was assigned to patients with overall scores > 7.

Sampling and analytical methodology

Plasma and serum were obtained from blood drawn from seated patients after a 12-hour overnight fast and at least 15 minutes after the placement of a peripheral intravenous catheter.

All lipid measurements were performed using a Hitachi 911 system (Roche Diagnostic Systems, Basel, Switzerland). Serum triglycerides were measured by a fully enzymatic standard method; high-density lipoprotein (HDL) cholesterol was measured by a direct method using polyethylene glycol- modified enzymes (PEGME)[17]. Low-density lipoprotein (LDL) cholesterol was measured after ultracentrifugation according to the method recommended by the Lipid Research Clinic. Insulin resistance was estimated by the homeostasis model assessment method (HOMA-IR) [18].

Plasma HIV-1 viral load was measured using the Amplicor HIV-1 Monitor assay (Roche Diagnostic Systems), which has a lower detection limit of 20 copies/ml.

Serum FGF19 and FGF21 levels were determined using non-cross-reactive enzyme linked immunoadsorbent assays (ELISAs) specific for the corresponding human proteins (Biovendor, Germany). Serum FGF19 data exhibited a normal distribution in patients and control population, whereas the distribution of serum FGF21 data distribution was skewed and was thus log-transformed before analysis, as described previously [11].

Biopsy samples of subcutaneous fat from ten patients from each patient group and ten healthy controls were taken from the abdominal area. After homogenization in RLT buffer (Qiagen, Hilden, Germany), RNA was isolated using a column-affinity based methodology that included on-column DNA digestion (RNeasy; Qiagen). One microgram of RNA was transcribed into cDNA using MultiScribe reverse transcriptase and random-hexamer primers (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, California, CA, USA). For quantitative mRNA expression analysis, TaqMan reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed on the ABI PRISM 7700HT sequence detection system (Applied Biosystems). The TaqMan RT-PCR reactions were performed in a final volume of 25 µl using TaqMan Universal PCR Master Mix, No AmpErase UNG reagent and primer pair probes specific for FGF21 (Hs00173927_m1), FGF19 (Hs00192780_m1), FGFR1 (Hs00222484_m1), β-Klotho (Hs00545621_m1), lactase-like protein (Lctl, Hs01385107_m1) and 18S rRNA, (Hs99999901). Controls with no RNA, primers, or RT were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA levels for the genes of interest. Values were normalized to that of the reference control (18S ribosomal RNA) using the comparative $2^{-\Delta CT}$ method, following the manufacturer's instructions. Parallel calculations performed using the reference gene PPIA (Hs99999904) yielded essentially the same results.

Statistical analysis

Data were expressed as means ± standard errors of the mean (SEMs), frequencies or percentages relative to healthy controls (defined as 100%). The normality of parameter distributions was determined using a Kolmogorov-Smirnov analysis. One-way analysis of

variance and Tukey post hoc tests were performed for comparisons of parametric data. Regression analysis was used to determine the linear relationships of anthropometric, metabolic and antiretroviral treatment variables with serum FGF19 quantitative parameters

Statistical analyses were performed using the Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, IL) and the SAS version 9.1.3 software (SAS Institute Inc., Cary, NC), P values < 0.05 (determined by two-sided tests) were considered significant.

RESULTS

A total of 106 HIV-1-infected patients and 31 healthy controls were studied. Thirty-four of the HIV-1-infected patients had not received any antiretroviral treatment (naïve), whereas 72 were receiving HAART. Thirty-eight of the HIV-1-infected patients undergoing HAART had been diagnosed as having lipodystrophy. There were no statistically significant differences between patients and controls with respect to sex distribution. Cumulative exposure to NRTIs and non-NRTIs (NNRTIs) was significantly greater in patients with lipodystrophy than in those without lipodystrophy, but there were no significant differences with respect to protease inhibitors (PIs) exposure time (Table 1). Data on individual drug-exposure profiles of HAART are shown in Table 2, and indicated that the only significant difference between lipodystrophy-positive (LD+) and lipodystrophy-negative (LD-) patients was a significantly higher duration of exposure to stavudine and didanosine in the LD+ group.

With respect to anthropometric parameters, there were no significant differences in BMI between either of the two groups of HAART, patients and controls; however the waist-to-hip ratio was significantly higher in HAART patients. There was a small but significant decrease in BMI in "naïve" patients. Among parameters indicative of circulating lipid homeostasis, triglyceridemia was markedly increased in HIV-1-infected, HAART patients, whereas total cholesterol, HDL-cholesterol, and LDL cholesterol were significantly lower in all the HIV-1-infected patient groups compared to healthy controls. Blood glucose levels were increased in both HIV-1-infected subsets, HAART patients compared to controls, and the increase in glycemia was significantly higher in those with lipodystrophy than in those without. The same pattern was observed for plasma insulin: its levels were increased in HIV-1-infected compared with uninfected individuals, the greatest increase occurring in patients with lipodystrophy. The HOMA-IR index of insulin resistance was increased in both groups of HIV-1-infected, HAART patients, with the greatest increase occurring in those with lipodystrophy.

Serum FGF19 levels in patients and healthy controls ranged from 26.1 pg/ml to 64.1 pg/ml. There were no significant differences ($P = 0.89$) in serum FGF19 levels between men (217.9 $\pm$

12.1 pg/ml, n = 104) and women (214.8 ± 20.8, n = 33). Serum levels of FGF19 in all HIV-1-infected patient groups were significantly reduced relative to those in controls (Fig 1). Among HIV-1-infected patients, those undergoing HAART and presenting lipodystrophy had the lowest FGF19 levels, non-treated patients had the highest levels, and HAART patients without lipodystrophy had intermediate levels; however these differences among HIV-1-infected patient groups did not attain statistically significance.

Serum FGF21 levels were progressively and significantly increased from healthy controls to naïve HIV-1-infected patients, treated without lipodystrophy patients, and treated with lipodystrophy patients. FGF21 levels were significantly higher in all HIV-1-infected patient groups than in healthy controls, and HAART patients with lipodystrophy had significantly higher levels of FGF21 than HAART patients without lipodystrophy (Fig 1), in agreement with a previous report [11].

We investigated the relationship between serum FGF19 levels and anthropometric parameters, metabolic data (including FGF21 levels), and parameters related to HIV-1 infection and HAART patterns (Table 2). Serum FGF19 levels correlated positively with HDL cholesterol levels and negatively with insulin levels and HOMA-IR. We found no significant correlation with parameters indicative of overall fat mass, such as BMI or fat percentage, or in relation to fat distribution, such as waist-to-hip ratio. In fact, when the overall patient population and controls were considered together, there were not statistically significant differences in serum FGF19 levels between lean (216.6 ± 12.3 pg/ml , BMI < 25 kg/m^2, n = 93) and overweight (218.4 ± 19.6 pg/ml, BMI > 25 kg/m^2, n =44) subjects. Serum FGF19 levels correlated negatively with cumulative months of treatment with NRTI and NNRTI drugs but not with cumulative duration of PI exposure. When these correlations were investigated in relation to individual drug treatment patterns for NRTIs (Table 3), a statistically significant negative correlation was found for the extent of treatment with stavudine (R = –0.21, P = 0.015), lamivudine (R = –0.18, P = 0.037) and tenofovir (R = –0.20, P = 0.021). No significant correlation was found for cumulative treatment with other NRTI drugs present in the HAART regimes of the patients such as zidovudine (R = –0.02, P = 0.78), abacavir (R = -0.08, P = 0.36), or didanosine (R= –0.07; P = 0.39). For NNRTIs, only cumulative months of treatment with efavirenz showed a significant negative correlation with serum FGF19 levels (R = –0.19, P = 0.029). No significant correlation between serum FGF21 and serum FGF19 levels was found.

Given the profound -and opposite- alterations in the endocrine FGF system in HIV-1-infected patients, with the abnormally low serum levels of FGF19 found here and the high levels of FGF21 reported previously and confirmed here, we analyzed the expression of mRNA for receptors and co-receptors for these endocrine FGFs in adipose tissue from patients and controls, as well as the expression of the FGF19 and FGF21 mRNA. The FGF19 mRNA

expression was undetectable in adipose tissue from all patient groups, as expected [19]. In fact, FGF19 is known to be expressed mainly in the intestine, although under pathological conditions it may be expressed in ectopic sites, such as liver[20]. Similarly, little or no FGF21 mRNA expression was detected in subcutaneous adipose tissue from HIV-1-infected patients; FGF21 expression was also virtually undetectable in adipose tissue from healthy controls, in agreement with other previous reports[21]. Expression of the mRNA of FGFR1, the main FGF receptor expressed in adipose tissue known to mediate FGF19 and FGF21 effects, was significantly reduced in untreated HIV-1-infected patients and in HIV-1-infected, HAART patients with no lipodystrophy compared to controls (Fig 2). There was no significant difference in FGFR1 gene expression in adipose tissue between HIV-1-infected, HAART patients with lipodystrophy and controls. Next, we determined the mRNA expression levels for β-Klotho, the co-receptor of FGFR1 that determines the specificity of cell responsiveness to the FGF19 and FGF21 members of the FGF family. The results indicated a dramatic decrease in β-Klotho transcript expression in adipose tissue from all patient groups, a decrease that was specially marked in naive patients (Fig 2). The mRNA levels of Lctl, a recently described potential co-receptor for FGF19 signaling [22], were negligible in human adipose tissue from both controls and patients. In light of the reported role of PPARγ (peroxisome proliferator activated receptor-γ), the master regulator of adipogenesis, in β-Klotho gene regulation[23], as well as the known alterations in PPARγ gene expression in adipose tissue from HIV-1 infected patients[2], PPARγ mRNA levels were determined in the patient groups. Results indicated that PPARγ expression was significantly lowered in all patient groups (percent values respect to controls: 16 $\pm$ 2% in naïve patients, $P < 0.001$; 36 $\pm$ 3% in LD- patients, $P = 0002$; 14 $\pm$ 3% in LD patients, $P < 0.0001$), in agreement with previous reports.

Discussion

The present study demonstrates that serum FGF19 levels are reduced in HIV-1-infected patients. The fact that FGF19 levels were negatively correlated with parameters indicative of insulin resistance, such as HOMA-IR, is consistent with the proposed role of FGF19 in promoting insulin sensitivity and glucose uptake in target tissues based on experimental animal studies [5;20]. Although the cross-sectional nature of the present study does not allow cause-to-effect relationships, it may be speculated that the abnormally low levels of FGF19 in HIV-infected patients contribute to metabolic disturbances such as hyperglycemia and insulin resistance that occur frequently in these patients. The recent proposal indicating that pharmacological treatment of rodent models with FGF19 may boost certain effects of insulin on liver is also consistent with this scenario[8]. On the other hand, the lack of association of FGF19

levels and BMI in the HIV-1-infected population is in contrast with a recently reported inverse association in obese population[24]. It is likely that the narrow range of BMI in our HIV-1 patient population precluded detection of such association in contrast with the extremely wide range of BMI values in obesity studies.

Given the very limited knowledge about the regulation of FGF19 production and release, the mechanisms by which FGF19 levels are reduced in patients is a matter of speculation. The ileum is a main site of FGF19 production; in this part of the intestine, FGF19 production is induced by bile acids [20]. There is evidence that chenodeoxycholic acid increases FGF19 levels in volunteers, whereas treatment with the bile acid-binding resin cholestyramine decreases serum FGF19 [25]. To our knowledge, only one published study has assessed bile acid levels in HIV-infected patients[26]. This report describes distinct alterations in individual bile acid concentrations in HIV-1-infected patients, from a rise in the relative concentrations of lithocholic and taurocholic acids to a relative decrease in chenodeoxycholic acid. Further studies will be required to establish whether the reduction in the serum FGF19 levels in patients is a consequence of altered bile acid levels.

Despite the fact that levels of FGF19 were significantly reduced in patients before HAART, the present study demonstrated a negative correlation between FGF19 levels and the extent of the treatment of patients with NRTIs, particularly with stavudine, and with the NNRTI efavirenz. Stavudine is one of the components of anti-retroviral regimes that is more closely associated with adipose tissue alterations, including lipodystrophy [27]. Although we found no correlation between FGF19 levels and specific indicators of lipodystrophy, the lowest levels of FGF19 in our patients groups were observed in patients with lipodystrophy. On the other hand, efavirenz has not traditionally been associated with the appearance of signs of metabolic syndrome or lipodystrophy symptoms. However, an extensive randomized study (ACTG A5142) recently reported that lipoatrophy was more common with efavirenz than with PIs when combined with stavudine or zidovudine [28].

A major finding of the present study is the reduction in the expression of FGFR1 and β-Klotho -main mediators of FGF19 and FGF21 responsiveness in adipose tissue- in HIV-infected patients. This result –to our knowledge the first reported finding of altered gene expression for components of the endocrine FGF system responsiveness in human tissues- is strongly supportive of impaired responsiveness to endocrine FGFs. It is also consistent with the proposal that resistance to FGF21 accounts for the paradoxical increase of FGF21 in these patients in the absence of an improvement in glucose utilization and lipid handling that would normally be expected from exposure of tissues to high levels of FGF21. A similar scenario has been recently reported in rodent models of obesity, in which resistance to the effects of FGF21 is associated with down-regulation of FGF receptors and β-klotho in white fat [14]. For FGF19, the situation is

such that the beneficial metabolic effects of FGF19 would be strongly blunted in adipose tissue from patients by the combination of lowered levels of the hormone and reduced expression of its receptor system components. In fact, it has been recently proposed that beneficial metabolic effects of FGF19 apply mainly to its action in adipose tissue[7].

In light of the minimal available information about the regulation of FGF receptors and β-klotho co-receptor proteins, any proposed mechanisms to account for the reduction of their expression can only be speculative. However, because the gene expression for this proteins is markedly reduced HIV-1-infected patients even without HAART, it is likely that alterations caused by HIV-1 infection itself, ranging from inflammation to initial alterations in lipid metabolism, may contribute to abnormally low expression. Among the initial alterations reported in fat from HIV-1-infected patients before treatment, and as confirmed here, is the abnormal down-regulation of the transcription factor PPARγ[2,29]. This alteration appears to be the consequence of pro-inflammatory signals (e.g. enhanced tumor necrosis factor-α levels) and the direct action of HIV-1-encoded proteins such as Vpr [30]. It has been reported that β-Klotho is strongly regulated by the PPARγ activator rosiglitazone in cultured adipocytes [23], suggesting that abnormal PPARγ-dependent regulation of gene expression may account for the strong reduction in β-Klotho in adipose tissue from HIV-1-infected patients.

In summary, we have found that the levels of FGF19 are decreased and most probably the sensitivity to FGF19 and FGF21 is reduced in adipose tissue in HIV-1-infected patients. These alterations may attenuate the beneficial effects of endocrine FGFs in HIV-infected patients and contribute to their metabolic alterations. However, considering that, in experimental models of reduced expression of endocrine FGFs receptors, pharmacological doses of FGF21 still produce beneficial effects [4,13], the potential of FGF19 and/or FGF21 treatment to ameliorate metabolic alterations in HIV-1-infected patients undergoing HAART warrants future attention.

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Figure legends

Fig 1. **Serum levels of FGF19 and FGF21 in healthy controls, untreated (naïve) HIV-1 infected patients, and HAART patients with lipodystrophy (LD+) or without lipodystrophy (LD-).** Serum levels of FGF21 are log-transformed. Data are presented as mean ± SEM. *P* values < 0.05 for comparisons between groups are shown.

Figure 2. **FGFR1 and β-Klotho mRNA expression in adipose tissue from healthy controls, untreated (naïve) HIV 1-infected patients, and HAART patients with lipodystrophy (LD+) or without lipodistrophy (LD-).** Values are expressed relative to 18S rRNA levels and are means ± SEM of 8-10 patients/group. *P* values < 0.05 for comparisons between groups are shown.

Figure 1
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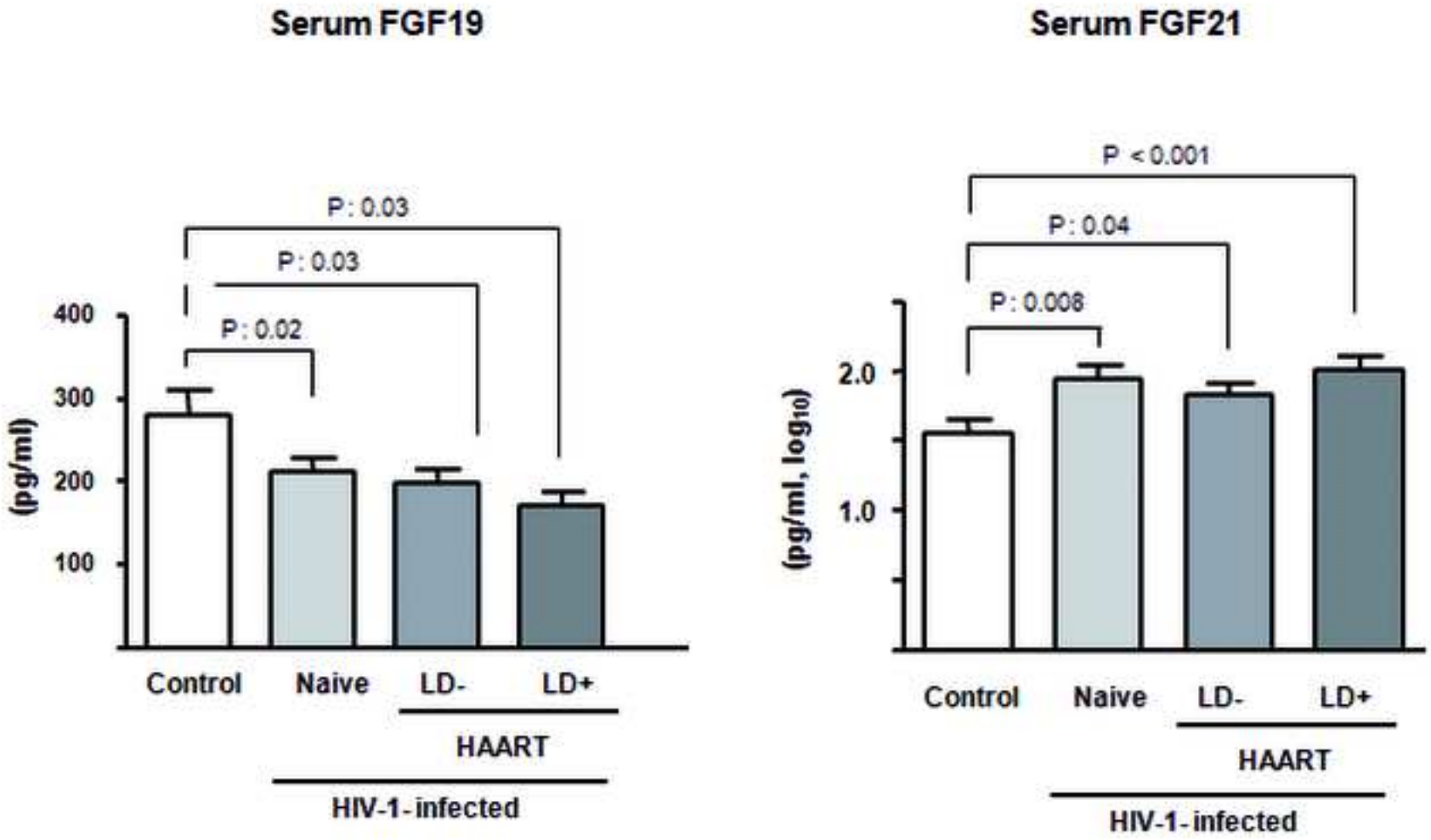

Figure 2
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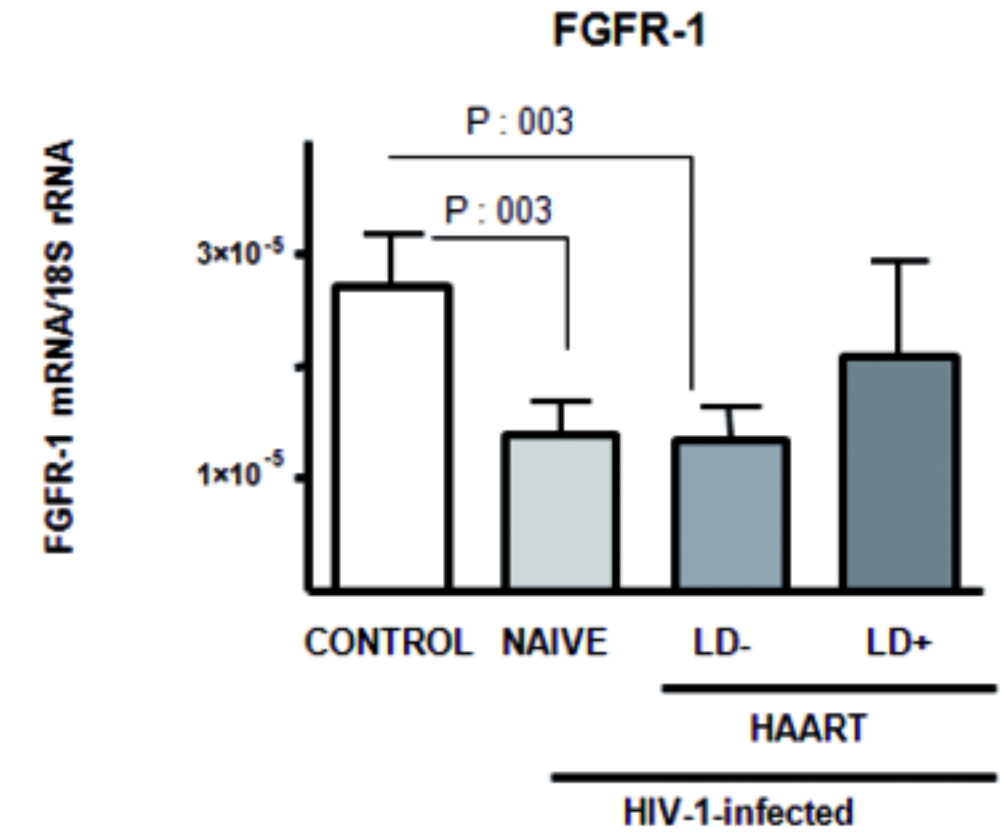

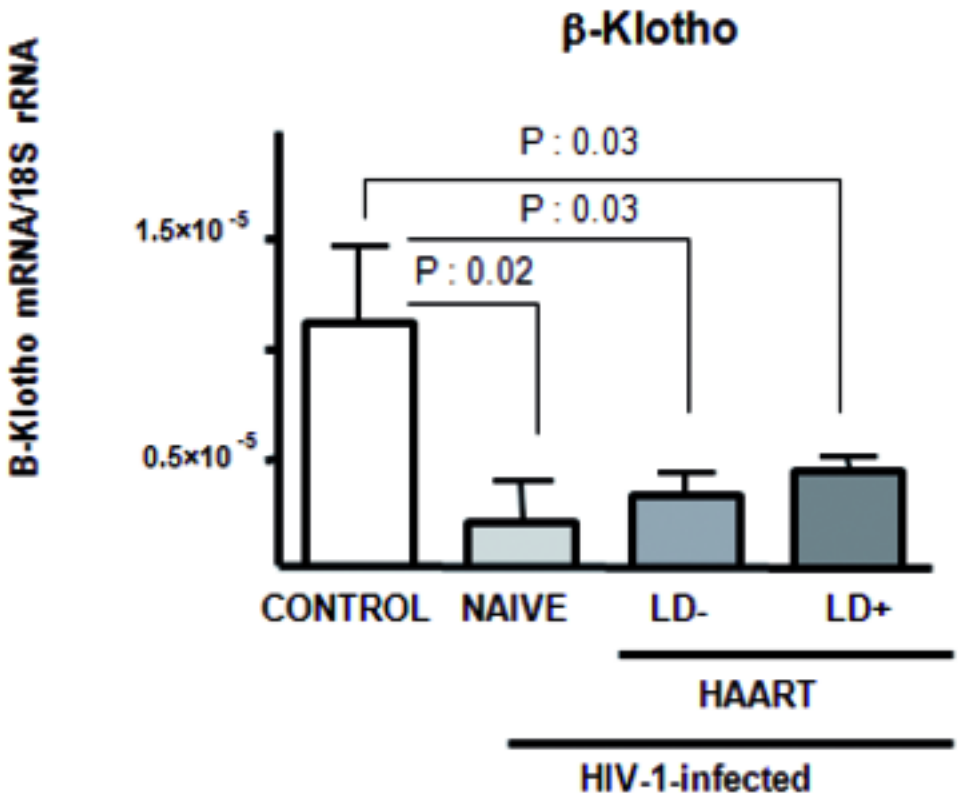

Table 1. Demographic, anthropometric, metabolic and HIV-1 infection and antiretroviral exposure parameters.

		HIV-1 infected patients									
	Control C (n=31)	Naive (n=34)	P *value* N vs C	LD- (n=34)	P *value* LD- vs C	P *value* LD-vs N	LD+ (n=38)	P *value* LD+vs C	P *value* LD+ vs N	P *value* LD+ vs LD-	*P Value*
Sex (n of men (%))	24 (77,4)	28 (82,3)	0,759	26 (76,5)	0,999	0,765	26 (68,4)	0,432	0,276	0,599	0,576
Age	43,77 ± 0,94	38,58 ± 1,81	**0,020**	43,41 ± 1,21	0,821	**0,032**	44,68 ± 1,11	0,542	**0,005**	0,439	**0,006**
BMI	24,87 ± 0,47	22,78 ± 0,62	**0,012**	23,65 ± 0,54	0,100	0,300	23,80 ± 0,57	0,172	0,232	0,847	0,095
Waist circunference (cm)	100,20 ± 1,24	93,17 ± 1,25	**0,001**	94,00 ± 1,23	**0,001**	0,638	92,08 ± 1,09	< **0.0001**	0,513	0,245	< **0.0001**
Waist-to-hip ratio	0,87 ± 0,01	0,89 ± 0,01	0,508	0,94 ± 0,02	**0,001**	**0,004**	0,94 ± 0,01	**0,001**	**0,004**	0,852	**0,001**
Total body fat (%)	24,88 ± 0,79	20,14 ± 1,30	**0,004**	20,69 ± 1,20	**0,006**	0,754	19,92 ± 1,02	**0,001**	0,895	0,622	**0,009**
Total cholesterol (mmol/l)	5,18 ± 0,17	4,30 ± 0,17	**0,001**	4,66 ± 0,14	**0,025**	0,104	5,04 ± 0,21	0,627	**0,009**	0,162	**0,003**
Triglycerides (mmol/l)	0,98 ± 0,08	1,27 ± 0,12	0,051	2,20 ± 0,28	**0,001**	**0,003**	2,37 ± 0,22	< **0.0001**	< **0.0001**	0,629	< **0.0001**
HDL cholesterol (mmol/l)	1,44 ± 0,05	1,12 ± 0,08	**0,003**	1,26 ± 0,07	**0,044**	0,199	1,21 ± 0,05	**0,003**	0,380	0,540	**0,011**
LDL cholesterol (mmol/l)	3,31 ± 0,15	2,72 ± 0,15	**0,009**	2,37 ± 0,12	< **0.0001**	0,068	2,85 ± 0,17	0,052	0,588	**0,025**	**0,001**
Glucose (mmol/l)	4,87 ± 0,07	4,99 ± 0,11	0,397	5,28 ± 0,12	**0,006**	0,067	5,50 ± 0,14	**0,001**	**0,008**	0,258	**0,001**
Insulin (pmol/l)	41,47 ± 4,61	40,83 ± 4,10	0,918	63,44 ± 8,44	**0,031**	**0,017**	112,60 ± 17,00	**0,001**	**0,001**	**0,015**	< **0.0001**
HOMA-R	0,79 ± 0,08	0,77 ± 0,07	0,874	1,18 ± 0,15	**0,033**	**0,016**	2,01 ± 0,25	< **0.0001**	< **0.0001**	**0,008**	< **0.0001**
SBP (mmHg)	121,00 ± 2,55	115,50 ± 1,67	0,069	121,50 ± 3,03	0,901	0,084	119,00 ± 2,75	0,604	0,291	0,543	0,339
DBP (mmHg)	71,17 ± 1,86	71,67 ± 1,45	0,831	76,71 ± 1,74	**0,034**	**0,029**	74,53 ± 1,32	0,135	0,149	0,316	0,054
AST (U/I)	23,88 ± 1,139	25,97 ± 2,153	0,432	33,03 ± 3,403	**0,029**	0,089	35,54 ± 6,555	0,153	0,196	0,741	0,196
ALT (U/I)	29,79 ± 3,341	31,64 ± 3,015	0,682	48,82 ± 10,57	0,120	0,128	53,18 ± 15,76	0,184	0,184	0,818	0,257
GGT (U/I)	41,04 ± 5,95	46,28 ± 9,082	0,645	93,41 ± 20,57	**0,027**	**0,037**	99,13 ± 22,99	**0,027**	**0,037**	0,854	**0,025**
Total bilirubin (Umol/l)	11,10 ± 1,33	14,42 ± 5,48	0,597	20,09 ± 3,97	**0,049**	0,410	10,81 ± 1,17	0,869	0,517	**0,023**	0,243
ALP (U/l)	86,66 ± 6,32	79,11 ± 5,75	0,381	89,35 ± 10,51	0,834	0,392	76,70 ± 4,45	0,190	0,740	0,258	0,533
FGF21 (pg/ml, log10)	1,56 ± 0,09	1,94 ± 0,10	**0,008**	1,83 ± 0,09	**0,039**	0,401	2,01 ± 0,09	**0,001**	0,611	0,150	**0,006**
Plasma viral load (log10)		4,19 ± 0,19		2,11 ± 0,16		< **0.0001**	2,12 ± 0,13		< **0.0001**	0,953	< **0.0001**
CD4+ count (cels/mm^3)		468,9±32,87		665,0±48,54		**0,001**	665,4±50,88		**0,002**	0,995	**0,003**
Antiretroviral drug exposure											
NRTI (months)				184,90 ± 9,87			220,70 ± 7,98				**0,002**
NNRTI (months)				33,56 ± 4,91			49,34 ± 4,67				**0,023**
PI (months)				50,81 ± 5,43			58,63 ± 5,69				0,225

Parameters are expressed as mean ± SEM unless especified. *P* values were calculated using one-way analysis of variance and Tukey post-test for parametric data. Bold lettering is shown when P < 0,05. ALP, alkaline phosphatase, ALT. Alanine amino transferase; AST, aspartate amino transferase; BMI, body mass indez, GGT, γ-glutamyl transferase; HDL, high-density lipoprotein, , HOMA-R, homeostasis model assessment of insulin resistance, LD-, no lipodystrophy; LD+, lipodystrophy; LDL, low-density lipoprotein, NRTI, nucleoside analog reverse transcriptase inhibitor; NNRTI, nonnucleoside analog reverse transcriptase inhibitor; PI, protease inhibitor.

Table 2. **Linear relationship of circulating FGF19 levels with demographic, anthropometric, metabolic, HIV-1 infection and antiretroviral exposure parameters**

	Serum FGF19	
	r	*P*
Age	0,01	0,911
BMI	0,04	0,677
Waist circunference	-0,05	0,597
Waist-to-Hip ratio	-0,09	0,288
Total body fat	-0,07	0,404
Total cholesterol	0,03	0,767
Triglycerides	-0,11	0,185
HDL cholesterol	0,17	**0,025**
LDL cholesterol	0,06	0,453
Glucose	-0,10	0,257
Insulin	-0,16	**0,046**
HOMA-IR	-0,19	**0,032**
SBP	-0,04	0,625
DBP	-0,11	0,212
AST	-0,07	0,442
ALT	-0,04	0,628
GGT	-0,03	0,764
Total bilirubin	-0,08	0,362
ALP	0,13	0,150
FGF21	-0,07	0,374
Plasma viral load	-0,06	0,557
CD4+ count (cels/mm^3)	-0,04	0,661
Antiretroviral drug exposure		
NRTI (months)	-0,20	**0,020**
NNRTI (months)	-0,23	**0,007**
PI (months)	-0,11	0,197

The correlations were calculated in the combined population of healthy controls and HIV-1 infected patients with or without antiretroviral-treatment. Units and abbreviations are as in Table 1. Statistical significance is from Pearson correlation test. Bold lettering is shown when $P < 0,05$.

Serum FGF21 levels are elevated in association with lipodystrophy, insulin resistance and biomarkers of liver injury in HIV-1-infected patients

Revista: AIDS. 2010 Nov 13; 24(17):2629-37.

PMID: 20935553

Título: Elevados niveles de FGF21 en suero asociados con lipoditrofia, resistencia a insulina y biomarcadores de daño hepático en pacientes infectados por HIV-1

Los pacientes infectados por HIV-1 con lipodistrofia presentan resistencia a insulina, dislipidemia y otras señales de síndrome metabolico. Se ha propuesto que el nuevo regulador metabólico FGF21 provoca efectos beneficiosos para la homeostasis metabolica y la sensibilidad a insulina. Nuestro objetivo en este artículo era determinar la relación entre los niveles de FGF21 y las alteraciones metabólicas en estos pacientes. Para ello se analizaron los niveles de FGF21 en 179 individuos que pertenecían a los siguientes grupos: 59 pacientes infectados por HIV-1 en tratamiento retroviral que han desarrollado lipodistrofia, 45 pacientes infectados por HIV-1 en tratamiento retroviral sin lipodistrofia, 41 pacientes infectados por HIV-1 no tratados y 34 controles sanos. Los niveles de FGF21 de estos individuos se correlacionaron con parámetros de distribución de grasa, riesgo cardiovascular y metabólico y con la infección por el virus HIV-1 así como con los diferentes medicamentos de la terapia HAART. Se observó que los niveles de FGF21 estaban aumentados en los pacientes infectados pero el aumento era más marcado en los pacientes con lipodistrofia. Así mismo, los niveles de FGF21 muestran una fuerte correlación con indicadores de lipodistrofia (como el ratio de grasa tronco/apendicular o el índice cintura-cadera), indicadores de resistencia a insulina (como niveles de glucosa en ayuno o HOMA-R), indicadores de dislipidemis (como colesterol LDL) e indicadores de daño hepático (como γ-glutamiltransferasa). Por lo tanto este artículo concluye que los niveles de FGf21 están aumentados en los pacientes infectados por HIV-1, especialmente en los que sufren lipodistrofia, y este aumento está asociado con la resistencia a insulina, síndrome metabólico y marcadores de daño hepático. Serán necesarios más estudios para determinar si este aumento de FGF21 en suero esta causado por una respuesta compensatoria o una resistencia a FGF21 y establecer el potencial de FGF21 como biomarcador de alteraciones metabólicas en pacientes infectados por HIV-1 bajo tratamiento retroviral.

Serum FGF21 levels are elevated in association with lipodystrophy, insulin resistance and biomarkers of liver injury in HIV-1-infected patients

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Objective: HIV-1-infected patients with lipodystrophy show insulin resistance, dyslipidemia and other signs of metabolic syndrome. Fibroblast growth factor-21 (FGF21) is a novel metabolic regulator that has been suggested to exert beneficial effects on metabolic homeostasis and insulin sensitivity. Our goal was to determine the relationship between FGF21 levels and metabolic alterations in these patients.

Research design and methods: Serum FGF21 levels were analyzed in 179 individuals belonging to four groups: HIV-1-infected, antiretroviral-treated patients that have developed lipodystrophy ($n = 59$); HIV-1-infected, antiretroviral-treated patients without lipodystrophy ($n = 45$); untreated (naive) HIV-1-infected patients ($n = 41$); and healthy control individuals ($n = 34$). Serum FGF21 levels were correlated with parameters indicative of altered fat distribution, metabolic and cardiovascular risk, and in relation to HIV-1 infection and antiretroviral treatment regimens.

Results: Serum FGF21 levels were increased in all HIV-1-infected patients, but the increases were most marked in those with lipodystrophy. FGF21 levels showed a strong positive correlation with indicators of lipodystrophy (trunk/apendicular fat ratio, waist-to-hip ratio), insulin resistance (fasting glucose, HOMA-R), dyslipidemia (low-density lipoprotein cholesterol), and liver injury (γ-glutamyltransferase).

Conclusions: FGF21 levels are increased in HIV-1-infected patients, especially in those with lipodystrophy, and this increase is closely associated with insulin resistance, metabolic syndrome and makers of liver damage. Further research will be required to determine whether the increase in FGF21 levels is caused by a compensatory response or resistance to FGF21, and to establish the potential of FGF21 as a biomarker of altered metabolism in HIV-1-infected, antiretroviral-treated patients.

AIDS 2010, **24**:2629–2637

Keywords: antiretroviral drugs, FGF21, insulin resistance, lipodystrophy, metabolic syndrome

Introduction

Since the introduction of highly active antiretroviral therapy for HIV-1-infected patients, a subset of treated patients has been shown to develop the so-called lipodystrophy syndrome. This syndrome is characterized by altered distribution of adipose tissue, mainly lipoatrophy in peripheral areas (e.g. face, arms, buttocks), visceral

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Received: 12 April 2010; revised: 6 August 2010; accepted: 23 August 2010.

DOI:10.1097/QAD.0b013e3283400088

obesity and, sometimes, lipomatosis. This redistribution is associated with profound systemic metabolic alterations, such as insulin resistance and dyslipidemia, similar to the metabolic syndrome [1]. These metabolic disturbances are of complex origin, and their development may be affected by antiretroviral drugs as well as the underlying HIV-I infection [2]. Nucleoside analog reverse transcriptase inhibitors (NRTIs) used to treat HIV-1 infection are particularly associated with the lipoatrophy in subcutaneous fat [3], whereas protease inhibitors are considered more likely to cause systemic metabolic alterations such as insulin resistance [4]. Non-nucleoside-analog reverse transcriptase inhibitors (NNRTIs) are not thought to contribute to the development of lipodystrophy, although recent data have led to a reconsideration of the effects of some of these drugs on peripheral fat accumulation [5]. Metabolic alterations in patients with HIV-1 lipodystrophy lead to an increased risk of developing type II diabetes and to increased cardiovascular risk. There have been attempts to treat HIV-1-lipodystrophy using drugs of known effects against dyslipidemia (fibrates) or insulin resistance (thiazolidinediones), but results on the overall lipodystrophy syndrome have been poor [6,7].

Fibroblast growth factor-21 (FGF21) is a member of the FGF family of proteins. It is produced mainly by the liver, and has endocrine and paracrine actions. Recent studies using animal models have recognized FGF21 as a novel actor in metabolic regulation. Initially, FGF21 was reported to promote glucose utilization by extrahepatic tissues [8], and correct glucose intolerance and hyperlipidemia in rodent models of obesity and type II diabetes [9,10]. These beneficial effects have led to an increased interest in the potential of FGF21 as a treatment for insulin resistance specifically, and metabolic syndrome generally. In rodent models, FGF21 has been proposed to mediate the metabolic response to starvation, favoring lipolysis in adipose tissue and fatty acid oxidation and ketogenesis in the liver [11,12] and to promote brown fat thermogenic activity [13]. It has been shown that FGF21 expression in the liver responds to high fatty acid availability [14] and is under the control of PPARα, which mediates transcriptional activation of the *FGF21* gene in response to fatty acids [11,12]. Limited data from recent human studies on FGF21 regulation indicate that FGF21 levels are increased in response to starvation only after long periods of food deprivation [15] and are not closely associated with ketosis [16]. However, treatment with fibrates induces an increase in serum FGF21 levels, supporting the PPARα dependence of *FGF21* gene in humans [15,16], as has been demonstrated in rodents. Finally, there have been several recent independent reports that FGF21 levels are increased in obese and type II diabetic patients, and that FGF21 levels are associated with the metabolic syndrome in these patients [17–19].

In the present study, we have analyzed FGF21 levels in HIV-1-infected patients (with or without lipodystrophy) undergoing antiretroviral treatment, HIV-1-infected patients prior to drug treatment, and healthy controls. The results reveal a close association between changes in FGF21 levels and anthropometric signs of lipodystrophy, metabolic syndrome and hepatic damage.

Materials and methods

All patients were recruited through the same HIV-1 infection clinic at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain), and were consecutive patients with an established diagnosis of HIV-1 infection on treatment. Patients were eligible if they had either or not lipodystrophy and were on antiretroviral therapy or not. Patients who were hospitalized or have a frank cognitive impairment such as delirium or dementia on enrolment were not eligible. Patients with opportunistic infections, acute hepatitis, liver insufficiency, neoplasms or fever of undetermined origin were excluded from the study too. At the time of study entry no patient used any other drug known to influence glucose metabolism or fat distribution such as anabolic hormones or systemic corticosteroids, recombinant human growth hormone, or appetite stimulants. Informed consent was obtained from the patients at study entry. Controls were recruited among hospital personnel and had to be negative for HIV-1 infection and be between 35 and 45 years of age with a proportion of men of about 70%. To be eligible they did not have to meet any of the exclusion criteria used for patients. The study was approved by the Ethics Committee of the Hospital de la Santa Creu i Sant Pau.

We measured serum FGF21 levels in 145 HIV-1-infected patients and 34 healthy controls. Patient demographics, HIV-1 infection status and antiretroviral treatment parameters are shown in Table 1; anthropometric and metabolic data are shown in Table 2. The diagnosis of AIDS was based on the 1993 revised case definition of the Centers for Disease Control and Prevention (CDC) [20]. For body composition measurements, patients were weighed on calibrated scales after removing shoes, outdoor clothing, and other heavy items. Body mass index (BMI) was calculated, and waist circumference was measured to the nearest millimeter using anatomical landmarks, as defined by the Third National Health and Nutrition Evaluation Survey [21]. On the basis of their BMI, patients were classified as lean (BMI $< 25\,kg/m^2$), overweight (BMI $25–29.9\,kg/m^2$) or obese (BMI $\geq 30\,kg/m^2$). Whole body dual energy X-ray absorptiometry (DEXA) scans (Hologic QDR-4500A; Hologic, Inc, Waltham, Massachusetts, USA) were conducted by a single operator. The percentage of fat in the arms, legs and central abdomen (calculated from the mass of fat versus lean and bone mass) as well as total lean body mass (in kilograms) was recorded.

Table 1. Demographics, HIV-1 infection and antiretroviral exposure parameters.

	Controls ($n=34$)	HIV-1-infected patients			P value
		Naive ($n=41$)	LD− ($n=45$)	LD+ ($n=59$)	
Age (years)	42 (40–48.5)	35 (31–41)	43 (39–49)	45 (41–49)	<0.0001
Sex [no. of men (%)]	24 (75.0)	35 (85.4)	35 (77.8)	43 (72.9)	0.5082
Years of infection		2 (1–2)	11 (7–17)	14 (10–16)	<0.0001
AIDS [*n* (%)]		1 (2.4)	15 (33.3)	29 (49.2)	<0.0001
CD4 cell count		438 (342–611)	645 (408–897)	557 (351–844)	0.0440
Plasma viral load ($\log_{10}$)		4.387 (3.643–5.104)	2.013 (1.279–2.528)	2.053 (1.279–2.842)	<0.0001
Antiretroviral drug exposure					
NRTI (months)			193.5 (139.5–235)	228 (193–266)	0.0036
NNRTI (months)			34 (0–50)	51 (25–71)	0.0002
PI (months)			44 (21–69)	48 (27–77)	0.2787

Parameters are expressed as median (interquartile range) unless specified. NRTI, nucleoside analog reverse transcriptase inhibitor; NNRTI, non-nucleoside analog reverse transcriptase inhibitor; PI, protease inhibitor.

Lipodystrophy was assessed using a lipodystrophy severity grading scale (LSGS) based on that reported by Lichtenstein [22]. The degree of lipoatrophy and diffuse fat accumulation at each region was rated as absent (score: 0), mild (noticeable on close inspection; score: 1), moderate (readily noticeable by patient/physician; score 2), or severe (readily noticeable to a casual observer; score: 3). The overall score was the mean of the sum of the scores given by the patient and physician for fat loss or fat accumulation. A clinical diagnosis of lipodystrophy was assigned to patients with overall scores above 7. Metabolic syndrome was defined according to the US National Cholesterol Education Program (NCEP) Adult Treatment Panel III Guidelines, modified as recommended in the latest American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement [23]. Metabolic syndrome was defined as having three or more of the following metabolic risk factors: central obesity (waist circumference $\geq$80 cm in women and $\geq$90 cm in men); hypertriglyceridemia (fasting triglycerides $\geq$1.69 mmol/l); low HDL-cholesterol (fasting HDL $<$ 1.29 mmol/l in women and $<$1.04 mmol/l in men); hyperglycemia (fasting glucose $\geq$5.6 mmol/l or already taking oral hypoglycaemic agents for treatment of type II diabetes); and hypertension (sitting blood pressure $\geq$130/85 mm Hg, determined as the mean of two readings taken after resting for at least 10 min, or taking regular antihypertensive medications).

Serum was obtained from blood drawn from seated patients after a 12-h overnight fast and at least 15 min after the placement of a peripheral intravenous catheter. Patients were seated during blood drawing. All lipid measurements were performed using a Hitachi 911 system (Roche Diagnostic Systems, Basel, Switzerland). Serum triglycerides were measured by a fully enzymatic standard method; HDL-cholesterol was measured by a direct method using polyethylene glycol-modified enzymes (PEGMEs) [24]. Low-density lipoprotein (LDL)-cholesterol was measured after ultracentrifugation according to the method recommended by the Lipid Research Clinic, but HDL-cholesterol was measured using the PEGME method instead of precipitation. Insulin resistance was estimated by the homeostasis model assessment method (HOMA-R) [25]. Serum FGF21 levels were determined in duplicate for each sample using an ELISA specific for human FGF21 (Biovendor, Germany). Serum HIV-1 RNA concentrations (viral load) were measured using the Amplicor HIV-1 Monitor assay (Roche Diagnostic Systems), which has a lower detection limit of 20 copies/ml.

Data are expressed as median with interquartile range (percentile 25–percentile 75) and frequencies and percentages. Serum FGF21 data distribution was skewed and it was log-transformed before analysis, as in previous studies [18]. Categorical data were compared by use of Fisher's exact test. Continuous data were analyzed by means of a nonparametric analysis of variance by applying a rank transformation on the dependent variable; pairwise comparisons were adjusted by the Bonferroni's method.

The linear relationship of log-transformed anthropometric, metabolic and antiretroviral treatment variables with log-serum FGF21 were assessed using raw and adjusted standardized regression estimates which are equivalent to the Pearson correlation for the univariate analyses.

The analysis was performed using the Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, Illinois, USA) and the SAS version 9.1.3 software (SAS Institute Inc., Cary, North Carolina, USA), and the level of significance was established at the 0.05 level (two-sided).

Results

A total of 145 HIV-1-infected patients and 34 healthy controls were studied. Forty-one of the HIV-1-infected patients had not received any antiretroviral treatment

Table 2. Anthropometric and metabolic data.

		HIV-1-infected patients				
	Control (C) ($n=34$)	Naive (N) ($n=41$)	LD− ($n=45$)	LD+ ($n=59$)	*P*	Bonferroni adjusted significant pairwise comparisons
BMI	24.4 (23.6–26.0)	22.7 (20.4–25.4)	23.4 (21.1–26.5)	23.7 (21.6–26.3)	0.1533	–
Waist circumference (cm)	89.5 (82–92)	83 (75–90)	87 (82–95)	86 (80–97)	0.0413	–
Waist-to-hip ratio	0.88 (0.84–0.91)	0.87 (0.83–0.94)	0.932 (0.89–0.98)	0.93 (0.90–1.01)	<0.0001	C vs. LD+, C vs. LD−, N vs. LD+ N vs. LD−
Total body fat (%)	24.7 (21.9–27.7)	16.8 (14.7–24.0)	22.7 (17.1–26.9)	19.5 (13.9–25.3)	<0.0001	C vs. N, C vs. LD+, C vs. LD−
Trunk-to-apendicular fat ratio	1.15 (0.87–1.34)	1.02 (0.93–1.31)	1.36 (1.09–2.20)	2.42 (1.79–3.10)	<0.0001	C vs. N,C vs. LD+,C vs. LD−,N vs. LD+,N vs. LD−
Total cholesterol (mmol/l)	5.20 (4.31–5.77)	3.99 (3.67–5.03)	4.9 (4.23–5.20)	5.07 (4.11–5.94)	0.0006	C vs. N,N vs. LD+
Triglycerides (mmol/l)	0.83 (0.65–1.06)	0.95 (0.73–1.56)	2.03 (1.30–2.41)	2.06 (1.24–3.03)	<0.0001	C vs. LD+,C vs. LD−,N vs. LD+,N vs. LD−,
HDL cholesterol (mmol/l)	1.49 (1.19–1.62)	1.03 (0.83–1.31)	1.21 (1.01–1.46)	1.19 (0.98–1.35)	0.0003	C vs. N,C vs. LD+,C vs. LD−
LDL cholesterol (mmol/l)	3.27 (2.47–4.01)	2.41 (2.03–3.42)	2.47 (1.98–2.93)	2.84 (2.24–3.31)	0.0040	C vs. N,C vs. LD−
Glucose (mmol/l)	4.8 (4.5–5.2)	5.0 (4.6–5.4)	5.2 (4.8–5.5)	5.5 (5.1–6.1)	<0.0001	C vs. LD+,C vs. LD−,N vs. LD+,N vs. LD−
Insulin (pmol/l)	29 (20–65)	27 (20–48)	45 (30–83)	88 (58–138)	<0.0001	C vs. LD+,N vs. LD+,N vs. LD−,LD+ vs. LD−
HOMA-R	0.5 (0.4–1.2)	0.5 (0.4–0.9)	0.8 (0.6–1.6)	1.7 (1.5–2.6)	<0.0001	C vs. LD+,N vs. LD+,N vs. LD−,LD+ vs. LD−
SBP (mmHg)	118 (111.5–128)	120 (110–120)	120 (110–130)	120 (110–130)	0.3468	–
DBP (mmHg)	68 (62–76)	70 (68–80)	78 (70–82)	80 (70–80)	0.0068	C vs. LD+,C vs. LD−
Metabolic syndrome [*n* (%)]	5 (15.6)	1 (2.4)	13 (28.9)	21 (35.6)	0.0006	N vs. LD+, N vs. LD−
AST (U/l)	19 (16–23)	26 (20–31)	25 (19–35)	31 (24–53)	<0.0001	C vs. LD+,C vs. LD−
ALT (U/l)	23 (15–26)	24 (18–37)	31 (21–44)	34.5 (24–67)	0.0002	C vs. LD+,C vs. LD−,N vs. LD+
GGT (U/l)	20 (14–30)	18 (15–26)	40 (22–118)	73 (43–128)	<0.0001	C vs. LD+,C vs. LD−,N vs. LD+,N vs. LD−,LD+ vs. LD−

Parameters are expressed as median (interquartile range) unless specified. *P* values between individual groups are shown when less than 0.05. ALT, alanine amino transferase; AST, aspartate amino transferase; BMI, body mass index; GGT, γ-glutamyl transferase; HDL, high-density lipoprotein; HOMA-R, homeostasis model assessment of insulin resistance; LD−, no lipodystyrophy; LD+, lipodystrophy; LDL, low-density lipoprotein.

(naive), whereas 104 had received highly active antiretroviral therapy. Fifty-nine of the HIV-1-infected patients under antiretroviral treatment had been diagnosed with lipodystrophy. Patient demographics, HIV-1 infection parameters and antiretroviral treatment data are shown in Table 1. There were not statistically significant differences between patients and controls with respect to sex distribution. Mean duration of HIV-1 infection was 10.03 ± 10.0 years. Forty-five patients (31.0%) had had a prior AIDS-defining condition. Among the HIV-1-infected patient population, 11 patients (7.6%) were co-infected with hepatitis C virus, whereas 13 (8.9%) had chronic hepatitis B virus infection. Two patients were co-infected by both hepatitis viruses. Cumulative exposure to NRTIs and NNRTIs was significantly longer in patients with lipodystrophy than in those without lipodystrophy, but there were no significant differences with respect to protease inhibitor exposure time (Table 1). Data on individual drug-exposure profiles of antiretroviral-treated patients are shown in Supplemental Table 1, http://links.lww.com/QAD/A91, and indicated a significant higher time of exposure to stavudine and to didanosine in the LD+ group versus the LD− group and a marginally significant higher time of exposure to efavirenz (see Table 1, Supplemental Digital Content 1, http://links.lww.com/QAD/A91). Data on drug treatment pattern at the time of obtaining blood samples is shown in Table 2, Supplemental Digital Content 2, http://links.lww.com/QAD/A91.

Anthropometric and metabolic data are shown in Table 2. Among HIV-1-infected patients, 100 individuals (68.9%) had normal weight and 45 (31.1%) were overweight, whereas 22 control individuals (64.7%) had normal weight and 12 (35.3%) were overweight ($P = 0.85$). None of the patients or control individuals was obese.

Metabolic syndrome was diagnosed in 40 (22.3%) of the 179 individuals studied, based on NCEP criteria (see Materials and methods section). The percentage of patients with metabolic syndrome was highest in the lipodystrophy group, intermediate in the treated patients without lipodystrophy and lowest in the control and naive groups (Table 2).

In the analysis of serum FGF21 levels in the overall population of patients and healthy controls, two individuals showed levels below the detection limits of the assay (two healthy controls) and were excluded for further analysis. Serum FGF21 levels ranged from 12.5 to 2683.9 ng/l. There were no sex differences in serum FGF21 levels; the median was 66.2 ng/l (IQR 33.3; 174.1) in men ($n = 137$) and 55.9 ng/l (IQR: 25.7; 99.9) in women ($n = 40$) ($P = 0.0876$).

Serum FGF21 levels were progressively and significantly increased from healthy controls to naive, treated without lipodystrophy and treated with lipodystrophy patients

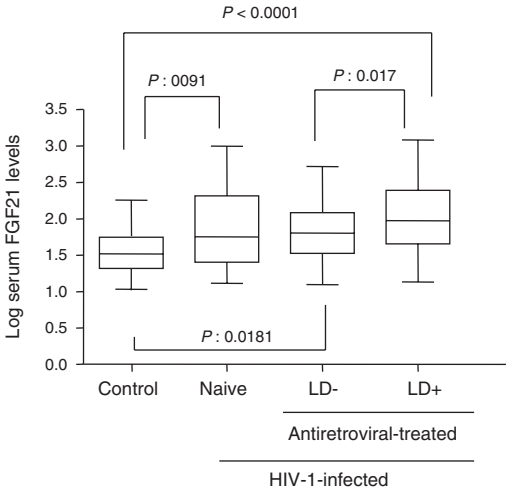

Fig. 1. Serum FGF21 levels in healthy controls, untreated HIV-1-infected patients (naive), and antiretroviral-treated patients with (LD+) or without (LD−) lipodystrophy. A box-and-whisker plot representing log-transformed serum FGF21 levels is shown. The line within the box marks the median, and the upper boundary of the box indicates the interquartile range. Error bars above and below the box denote the 100th and 0th percentiles, respectively. The *P* values are shown for comparisons between groups when less than 0.05.

(Fig. 1). FGF21 levels were significantly higher in all HIV-1-infected patient groups than in healthy controls, and treated patients with lipodystrophy had significantly higher levels of FGF21 than treated patients without lipodystrophy (Fig. 1).

Mean serum FGF21 levels in patients who had had AIDS were not significantly different with respect to patients without AIDS ($P = 0.33$). Co-infection with hepatitis C virus did not affect mean FGF21 serum levels ($P = 0.32$, for the comparison of FGF21 levels in HCV-positive patients versus HCV-negative patients).

We investigated the relationship between serum FGF21 levels and several anthropometric parameters, cardiometabolic risk factors and parameters related to HIV-1 infection and antiretroviral therapy patterns (Table 3). Serum FGF21 levels correlated positively with age, in agreement with previous reports [18]. FGF21 levels were positively correlated with waist-to-hip ratio and, in particular, the trunk/apendicular fat ratio (Table 3), both of which are markers of abnormal fat distribution; the correlation remained after adjusting for age. However, we found no significant correlation with respect to parameters indicative of overall fat mass, such as BMI or fat percentage. In fact, there were no statistically significant differences in serum FGF21 levels between lean (median: 56.4 ng/l; IQR: 26.7; 123.5) and overweight (median: 92.7 ng/l, IQR: 40.9; 245.9) individuals

Table 3. Regression standardized estimates for univariate and age-adjusted lineal relationship between serum log-transformed FGF21 levels and log-transformed anthropometric, metabolic and antiretroviral treatment parameters.

	Serum FGF21 (raw analysis)		Serum FGF21 (age-adjusted analysis)	
	Standardized estimate	*P*	Standardized estimate	*P*
Age	0.187	**0.013**		
BMI	0.035	0.639	−0.004	0.961
Waist circumference	0.103	0.172	0.053	0.493
Waist-to-hip ratio	0.259	**0.0005**	0.221	**0.005**
Fat percentage	−0.041	0.585	−0.074	0.326
Trunk/apendicular fat ratio	0.299	**<0.0001**	0.266	**0.0006**
Fasting glucose	0.221	**0.005**	0.174	**0.023**
Fasting insulin	0.251	**0.0008**	0.221	**0.004**
HOMA-IR	0.258	**0.0005**	0.227	**0.003**
Triglycerides	0.196	**0.009**	0.167	**0.028**
LDL-cholesterol	−0.169	**0.025**	−0.182	**0.015**
HDL-cholesterol	−0.070	0.354	−0.106	0.162
Systolic BP	0.175	**0.020**	0.144	0.055
Diastolic BP	0.106	0.160	0.105	0.159
AST	0.179	**0.017**	0.175	**0.018**
ALT	0.192	**0.011**	0.199	**0.007**
GGT	0.358	**<0.001**	0.337	**<0.001**
CD4 cell count	−0.050	0.550	−0.051	0.530
Plasma viral load (log_{10})	−0.009	0.912	0.090	0.312
Years of infection	0.127	0.134	0.414	0.646
NRTI exposure (m)	0.148	0.133	0.132	0.155
NNRTI exposure (m)	0.135	0.264	0.143	0.199
PI exposure (m)	0.012	0.990	−0.088	0.387

Serum FGF21 levels and independent variables were log transformed before analysis; m, months. Bold lettering is shown when $P < 0.05$. ALT, alanine amino transferase; AST, aspartate amino transferase; GGT, gamma-glutamyl transferase; NRTI, nucleoside analog reverse transcriptase inhibitor; NNRTI, nonnucleoside analog reverse transcriptase inhibitor; PI, protease inhibitor.

in the overall population of patients and controls ($P = 0.0813$).

Serum FGF21 levels correlated positively with fasting glucose, fasting insulin, the insulin resistance index (HOMA-R), triglycerides and LDL-cholesterol levels (Table 3). These positive correlations remained significant after adjusting for age for all factors. Among these parameters, HOMA-R achieved the highest level of statistical significance with respect to the correlation with serum FGF21 levels (Table 3). There was a marginally significant positive correlation between systolic BP and serum FGF21 levels, but significance was lost after adjusting for age. However, serum FGF21 levels were significantly higher ($P = 0.0013$) in patients with hypertension (median: 103.4 ng/l; IQR: 31.0; 153.8) ($n = 62$) than in those without hypertension (median: 51.5 ng/l; IQR: 16.7; 66.0) ($n = 60$).

Considering all the patients as a single population, serum FGF21 levels were significantly higher ($P = 0.0011$) in patients with the metabolic syndrome according to NECP criteria (median: 96.08 ng/l; IQR: 53.5; 253.1) ($n = 40$) than in individuals without metabolic syndrome (median: 57.3 ng/ml; IQR: 26.1; 132.5) ($n = 137$). Serum FGF21 levels showed a significant correlation with serum aspartate amino transferase (AST) and alanine amino transferase (ALT) levels whereas the correlation with gamma-glutamyl transferase (GGT) levels showed the highest statistical significance (Table 3). When patients with chronic hepatitis virus B or C infections were excluded from the analysis, significant correlation between FGF21 serum levels and GGT levels remained.

Finally, we investigated the relationship between serum FGF21 levels and HIV-1 infection and antiretroviral treatment parameters. FGF21 levels were not significantly correlated with CD4 cell count, plasma viral load or duration of exposure to NRTIs, NNRTIs or protease inhibitors.

Discussion

HIV-1 lipodystrophy is characterized by the combination of altered fat distribution and systemic metabolic alterations reminiscent of the metabolic syndrome (such as insulin resistance and dyslipidemia). These changes increase the risk of cardiovascular disease and the occurrence of type II diabetes in affected patients. Identifying agents capable of preventing the development of the syndrome and treated it once established is a challenge. FGF21 has been recently recognized as a novel metabolic regulator that favor glucose disposal in tissues and thus potentially exerts beneficial effects on the metabolic disturbances associated with type II diabetes.

Our present findings indicate that serum FGF21 levels are increased in HIV-1-infected patients with lipodystrophy. In fact, FGF21 levels were significantly increased in untreated HIV-1-infected patients, but the increase was much marked in HIV-1-infected, antiretroviral-treated patients that have developed lipodystrophy and thus show the greatest metabolic alterations. The highly significant correlation between FGF21 levels and indicators of altered fat distribution in lipodystrophy (such as trunk/apendicular fat ratio) is strong evidence for a positive relationship between FGF21 levels and the extent of lipodystrophy. The absence of a correlation between FGF21 levels and overall fat mass, in contrast with studies on obese patients [17,18], may reflect the relative narrow range of BMI values in our study population, which did not include obese patients.

The serum parameters most strongly correlated with FGF21 levels (HOMA-R, fasting glucose, LDL-cholesterol) were indicative of insulin resistance and metabolic syndrome. These findings are strongly similar to those reported in studies of obesity, in which increased FGF21 levels have been shown to be associated with metabolic syndrome [18]. Similar to findings from studies of obese patients, the high levels of FGF21 in patients with lipodystrophy appear paradoxical in light of the conventional view, based on experimental studies in rodents that FGF21 acts as an antidiabetic agent. The hypothesis offered to explain the observations in obese patients, resistance to FGF21 effects (as is known to occur for leptin) or an incompletely effective defense response to metabolic stress may also apply to HIV-1 lipodystrophy. In any case, obesity and HIV-1 lipodystrophy, two distinct abnormalities having in common the risk to develop the metabolic syndrome [2], display FGF21 increase as one more common, paradoxical, similarity.

The present findings indicate that patients treated with antiretroviral drugs had increased FGF21 levels even before developing lipodystrophy. However, correlative studies did not show any significant correlation between abnormal FGF21 levels and specific duration of treatment with NRTIs, or NNRTIs, or protease inhibitors. Moreover, naïve (previously untreated) HIV-1-infected patients showed a minor, but significant, increase in FGF21 levels compared with healthy controls. As for other disturbances in HIV-1-infected patients, it is likely that alterations caused by the HIV-1 infection itself, ranging from inflammation to initial alterations in lipid metabolism [26], may contribute to abnormally high initial levels of FGF21. Further research will be required to assess to what extent first alterations in FGF21 levels could predict in patients a deleterious outcome in relation to metabolic disturbances, given the strong association with lipodystrophy and insulin resistance in patients with full-blown lipodystrophy,

The present findings raise a question about the source of the abnormally high FGF21 levels in HIV-1-infected patients. Some studies have indicated that FGF21 is expressed in human adipose tissue [16,17] thus it may be that lipoatrophic subcutaneous fat or the enlarged visceral adipose tissue depots are sources of increased FGF21 levels. However, in humans, FGF21 expression in fat is around 100-fold lower than that in liver [17] (J.M. Gallego-Escuredo, F. Villarroya, unpublished observations), making it more likely that altered hepatic production of FGF21 is responsible for the high levels of FGF21 in the blood of HIV-1-infected patients. Moreover, rosiglitazone treatment and subsequent activation of PPARγ in adipocytes increases FGF21 expression [18], and HIV-1 lipodystrophy is associated with abnormal impairment, not induction, of the PPARγ pathway [26,27]. Liver function is markedly affected in HIV-1 lipodystrophy and the present findings indicate that the three biochemical markers of altered hepatic function, AST, ALT and GGT, were increased in HIV-1-infected patients under antiretroviral treatment, especially when they had developed lipodystrophy. Moreover, AST and ALT levels and specially GGT levels showed a significant positive correlation with the serum levels of FGF21. A positive correlation between FGF21 levels and biochemical markers of liver injury, especially GGT, has been reported in a recent study of Chinese patients with impaired glucose tolerance [28]. Because patients with biliary obstructive diseases, alcoholism and cirrhosis were excluded from the analysis and correlation remained after excluding the 16% patients co-infected with hepatitis C or hepatitis B viruses, the elevations of GGT in patients is likely to be associated with hepatic steatosis, reminiscent of the nonalcoholic fatty acid liver disease. In this sense, during the elaboration of the present study, Dushay *et al.* [29] reported that FGF21 expression is increased in the liver from patients with nonalcoholic hepatic steatosis. Multiple studies have indicated that antiretroviral-treated HIV-1 patients tend to develop hepatic steatosis, especially during the development of overt lipodystrophy [30–32]. In rodent models, lipid flow to the liver appears to be a primary metabolic determinant of hepatic FGF21 expression and release [11,12,14]. HIV-1 lipodystrophy is characterized by hyperlipidemia, and it is likely that enhanced availability and metabolism of fatty acids in the liver may cause increased hepatic FGF21 production, via PPARα activation. However, we cannot exclude the possibility that other as-yet-unidentified factors induced by HIV-1 infection and/or antiretroviral treatment cause abnormal induction of hepatic FGF21 synthesis and release, which in turn cause a high flow of fatty acids to liver due to the lipolytic action of FGF21 on adipose tissue. However, the action of FGF21 promoting adipose tissue lipolysis is still controversial [12,33,34].

The present study shows several limitations. The intrinsic cross-sectional nature of the study does not allow cause–effect relationships to be established between FGF21 and the metabolic parameters showing correlation with serum FGF21 levels. Moreover, further availability of data on

hepatic steatosis in patients would be needed to get insight into the relationship between FGF21 levels and altered hepatic enzymes. Overall, it appears that in HIV-1 lipodystrophy as in obesity and type II diabetes, it is yet unknown whether the increase in FGF21 levels enhances some of the metabolic alterations in metabolic syndrome, such as insulin resistance, or is merely a consequence of overall metabolic syndrome or altered hepatic metabolic function. As in obese, insulin-resistant patients, the FGF21 increase may also be explained by a compensatory response or resistance to FGF21. Moreover, as previously observed in rodent models of obesity and diabetes, high basal serum FGF21 levels do not preclude the potential use of FGF21 treatment to ameliorate insulin resistance and dyslipidemia. Further studies on the interplay between insulin, metabolic regulation and FGF21 will be needed to clarify these aspects in HIV-1-infected patients.

Acknowledgements

Thanks are given to F. Torres for support in statistical analysis.

Author contributions: P.D. and F.V. conceived and designed the study; J.G.-E. performed the FGF21 analyses; P.D. and F. Vidal recruited the patients and controls; M.M.G., M.G.M. and I.F. performed the analysis of anthropometric parameters and blood data. J.C.D. and M.G. coordinated the compilation, calculations and interpretation of data; P.D. and F.V. were responsible for final analysis of data and writing of the manuscript. All authors read and approved the final manuscript.

The study was supported by SAF2008-01896 from Ministerio de Ciencia e Innovación, grant PI081715 from the Instituto de Salud Carlos III, and Red de Investigación en SIDA, Instituto de Salud Carlos III (RD06/0006/0022 and RD06/0006/1004; Programa de Suport a Grups de Recerca AGAUR-Generalitat de Catalunya (2009 SR1061 and 2009 SR284) Spain.

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INFORME DEL DIRECTOR SOBRE LOS ARTÍCULOS PUBLICADOS

Informe dels directors en relació a la Tesi Doctoral de José Miguel Gallego Escuredo (publicacions i paper del doctorand)

En tant que directors de la Tesi doctoral de José Miguel Gallego Escuredo, fem constar que, a la memòria de la Tesi, els resultats obtinguts s'estructuren en set articles científics.

Articles científics que formen part de la Tesi

1) **Gallego-Escuredo JM**, Del Mar Gutierrez M, Diaz-Delfin J, Domingo JC, Mateo MG, Domingo P, Giralt M, Villarroya F.

Differential effects of efavirenz and lopinavir/ritonavir on human adipocyte differentiation, gene expression and release of adipokines and pro-inflammatory cytokines.

Curr HIV Res. 2010 Oct;8(7):545-53

Aquest primer article ha estat publicat a la revista *Current HIV Research.* Aquesta revista té un factor d'impacte, segons l'ISI, de 1.74, essent en el tercer quartil de l'àrea "Infectious Diseases". El doctorand, primer autor d'aquesta publicació, ha tingut tot el protagonisme en la obtenció experimental de les dades així com en l'evolució i progressió dels experiments, i ha participat amb gran protagonisme en la discussió dels resultats. El paper dels altres coautors ha estat el de investigadors sènior de l'àrea clínica que han participat fonamentalment en donar suport a alguns aspectes en el disseny i interpretació clínica de les dades i en algun altre cas donant suport logístc experimental.

2) Díaz-Delfín J, del Mar Gutiérrez M, **Gallego-Escuredo JM**, Domingo JC, Gracia Mateo M, Villarroya F, Domingo P, Giralt M.

Effects of nevirapine and efavirenz on human adipocyte differentiation, gene expression, and release of adipokines and cytokines.

Antiviral Res. 2011 Aug;91(2):112-9.

Aquest segon article ha estat publicat a la revista *Antiviral Research*. Aquesta revista té un factor d'impacte, segons l'ISI, de 4.30 essent en el primer quartil de les àrees "Virology" i "Pharmacology and Pharmacy".
Aquesta publicació té una orientació experimental i metodològica semblant a la anterior. En ella el doctorand ha participat fonamentalment donant suport experimental a mesures d'expressió gènica i de seguiment dels cultius cel·lulars d'adipòcits humans.

3) Guallar JP, **Gallego-Escuredo JM**, Domingo JC, Alegre M, Fontdevila J, Martínez E, Hammond EL, Domingo P, Giralt M, Villarroya F.

Differential gene expression indicates that 'buffalo hump' is a distinct adipose tissue disturbance in HIV-1-associated lipodystrophy.

AIDS. 2008 Mar 12;22(5):575-84.

Aquest tercer article ha estat publicat a la revista *AIDS,* Té un factor d'impacte 6.25 i es troba en el primer quartil de les àrees "Virology" , "Immunology" i "Infectious diseses". Es sense dubte la revista de més prestigi en el camp dels estudis sobre la SIDA, i la 4a revista de més alt impacte de l'àrea "Infectious diseases".
Dins aquest estudi de caracterització de teixit adipós en pacients, el doctorand contribuí en la realització de tota la bateria d'experiments sobre expressió de

proteïnes específiques per immunoblot, així com en la discussió i la obtenció de les conclusions del treball.

4) **Gallego-Escuredo JM**, Villarroya J, Domingo P, Targarona EM., Alegre M, Domingo JC, Villarroya F, Giralt M

Differential molecular signature of visceral adipose tissue alterations in HIV-1-associated lipodystrophy.

AIDS (submitted)

Aquesta treball ha estat sotmès per a la seva publicació a la revista *AIDS* (IF: 6.24), les característiques de la qual han estat esmentades anteriorment.
El doctorand, primer autor d'aquesta publicació, ha tingut la major part del protagonisme en la obtenció experimental de les dades, així com també ha contribuït a la discussió dels resultats. .

5) **José M. Gallego-Escuredo**, Pere Domingo, Joan Fontdevila, Joan Villarroya, Joan C Domingo, Marta Giralt, Francesc Villarroya

Hypertrophied facial fat in an HIV-1-infected patient after autologous transplantation from "buffalo hump" retains a partial brown fat-like molecular signature

Antivir Ther (Submitted)

Aquest article ha estat sotmés per a la seva publicació com a "Short Communication" a la revista *Antiviral Therapy*. Es una revista de molt relleu en l'ambit de recerca sobre el HIV, el tractament de la infecció i els seus efectes secundaris. Té un IF de 3.16 i és en el segon quartil de les àrees "Pharmacology and Pharmacy" , "Infectious Diseases" i "Virology"

El treball experimental ha estat realitzat majoritàriament pel doctorand, essent la contribució dels altres autors l'aportació de les mostres, dades analítiques clíniques i interpretació de dades des del punt de vista clínic, així com suport a algunes tecnologies analítiques específiques.

6) Domingo P, **Gallego-Escuredo JM,** Domingo JC, Gutiérrez Mdel M, Mateo MG, Fernández I, Vidal F, Giralt M, Villarroya F.

Serum FGF21 levels are elevated in association with lipodystrophy, insulin resistance and biomarkers of liver injury in HIV-1-infected patients.

AIDS. 2010 Nov 13;24(17):2629-37

Aquest article ha estat publicat a la revista *AIDS* (IF: 6.24), les característiques de la qual han estat esmentades anteriorment.
El doctorand, segon autor d'aquesta publicació, ha tingut tot el protagonisme en la obtenció experimental de les dades. Així mateix ha participat amb gran protagonisme en la discussió dels resultats. El paper dels altres coautors, i especialment del primer autor, ha estat en bon part el de proporcionar tota la bateria de mostres de pacients i totes les dades clíniques imprescindibles per al interpretació dels resultats des del punt de vista clínic i biomèdic.

7) **José M Gallego-Escuredo**, Pere Domingo , Maria del Mar Gutiérrez , Maria G. Mateo, Maria C Cabeza, Angels Fontanet, Francesc Vidal, Joan Carles Domingo, Marta Giralt, Francesc Villarroya

Reduced levels of serum FGF19 and impaired expression of receptors for endocrine FGFs in adipose tissue from HIV-infected patients

JAIDS (Acceptat, Agost 2012)

La publicació *JAIDS (Journal of Acquired Immune Deficiency Syndromes),* es una de les millors en el camp d'estudis sobre la SIDA. Té un factor d'impacte de 4.42, essent en el primer quartil de les àrees de "Immunology" i "Infectious Diseases"
El rol del doctorand, primer autor d'aquesta publicació, ha estat aquí també totalment protagonista en l'obtenció de les dades experimentals, el disseny i interpretació de les dades. Altes co-autors han participat en aspectes globals d'interpretació i disseny de l'estudi, així mateix, o bé en proporcionar tota la bateria de mostres de pacients i totes les dades clíniques imprescindibles per al interpretació dels resultats.

Finalment, cal esmentar que, donat el caràcter traslacional, multidisciplinar i de col.laboració en xarxa de la recerca al nostre grup sobre la temàtica de les patologies metabòliques presents en els pacients infectats pel HIV (treballs en el context de la RETIC SIDA i altres), durant la realització de la seva Tesi Doctoral José M. Gallego-Escuredo ha participat en diversos altres treballs que han estat publicats. Sovint ha realitzat aportacions experimentals puntuals necessàries per a aquest treballs, de temàtica estretament relacionada amb la seva Tesi, tot i no constituir el nucli d'aquesta. Aquests treballs son reportats al final de la memòria en forma d'apèndix.

A Barcelona, el 3 de setembre de 2012
Signat:

Francesc Villarroya i Gombau Marta Giralt Oms

Director Directora

ÍNDEX

APENDIX

A study of fatty acid binding protein 4 in HIV-1 infection and in combination antiretroviral therapy-related metabolic disturbances and lipodystrophy

Revista: HIV Medicine. 2011 Aug; 12(7):428-37. doi: 10.1111/j.1468-1293.2010.00903.x. Epub 2011 Jan 19.

PMID: 21251185

Título: Estudio de FABP-4 en pacientes infectados por HIV-1 tratados con terapia HAART con alteraciones metabólicas y lipodistrofia

El objetivo de este estudio era determinar lo niveles circulantes de FABP-4 ("Fatty Acid Binding Protein 4") en una población de pacientes infectados por HIV-1 tratados con terapia HAART, e investigar las relaciones entre los niveles de FABP-4 y la resistencia a insulina, dislipidemia, lipodistrofia y niveles de adipoquinas pro-inflamatorias en estos pacientes. Los niveles plasmáticos de FABP-4 eran significativamente superiores en pacientes con lipodistrofia que en los que no la sufrían. La concentración de FABP-4 se correlacionaba positivamente con BMI, HOMA-IR y concentraciones de insulina, colesterol total, triglicéridos, TNF-R1, leptina y IL-18. Por otra parte presentaba una correlación negativa con el colesterol HDL y concentración de adiponectina. Tras ajustar los resultados por edad, sexo y BMI, el ratio de "odds" para el riesgo de lipodistrofia resultó significativamente elevado para los pacientes con niveles más altos de FABP-4. En un modelo de regresión paso por paso, FABP-4 fue independientemente asociado con HOMA-IR tras controlar los parámetros clínicos e inflamatorios. Además se observó una relación positiva en pacientes con lipodistrofia entre la expresión de CD68 en el tejido adiposo subcutáneo y los niveles plasmáticos de FABP-4. Por lo tanto se concluye que los pacientes infectados por HIV_1 tratados con terapia HAART tienen una superproducción sistémica de FABP-4 que se asocia a la resistencia a insulina y marcadores de inflamación en el tejido adiposo subcutáneo.

DOI: 10.1111/j.1468-1293.2010.00903.x
HIV Medicine (2011), **12**, 428–437

ORIGINAL RESEARCH

A study of fatty acid binding protein 4 in HIV-1 infection and in combination antiretroviral therapy-related metabolic disturbances and lipodystrophy

X Escoté,[1,2*] A Megia,[1,2*] M López-Dupla,[1] M Miranda,[1,2] S Veloso,[1] V Alba,[1] P Domingo,[3] P Pardo,[4] C Viladés,[1] J Peraire,[1] M Giralt,[5] C Richart,[1,6] J Vendrell[1,2] and F Vidal[1] for the HIV-1 Lipodystrophy Study Group[†]

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Objective

The aim of the study was to determine circulating levels of fatty acid binding protein 4 (FABP-4) in a cohort of HIV-1-infected patients treated with combination antiretroviral therapy (cART) and to investigate the relationships between FABP-4 levels and insulin resistance, dyslipidaemia, lipodystrophy and levels of proinflammatory adipocytokines in these patients.

Methods

A total of 282 HIV-1-infected patients treated with stable cART for at least 1 year (132 with lipodystrophy and 150 without) and 185 uninfected controls (UCs) were included in the study. Anthropometric parameters were determined. Plasma levels of FABP-4, soluble tumour necrosis factor receptors 1 and 2 (sTNF-R1 and sTNF-R2), interleukin-18 (IL-18), IL-6, adiponectin and leptin were also analysed. Insulin resistance was determined using the homeostasis model assessment of insulin resistance (HOMA-IR). Subcutaneous adipose tissue mRNA expression of proinflammatory cytokines was assessed in 38 patients (25 with lipodystrophy and 13 without) by real-time polymerase chain reaction (PCR).

Results

The plasma FABP-4 concentration was significantly higher in patients with lipodystrophy than in those without ($P = 0.012$). FABP-4 concentration was positively correlated with body mass index (BMI), HOMA-IR, and the concentrations of insulin, total cholesterol, triglycerides, sTNF-R1, leptin and IL-18, but showed a negative correlation with high-density lipoprotein (HDL) cholesterol and adiponectin concentrations. After adjusting for age, sex and BMI, the odds ratio (OR) for risk of lipodystrophy was found to be significantly increased for those with the highest levels of FABP-4 [OR 0.838, 95% confidence interval (CI) 0.435–1.616 for medium FABP-4 *vs.* OR 2.281, 95% CI 1.163–4.475 for high FABP-4]. In a stepwise regression model, FABP-4 was independently associated with HOMA-IR after controlling for clinical and inflammatory parameters ($P = 0.004$). Moreover, a positive relationship was observed in patients with lipodystrophy between subcutaneous adipose tissue *CD68* expression and FABP-4 plasma levels ($r = 0.525$; $P = 0.031$).

*Contributed equally to this work.

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[†]See Appendix for group members.

Conclusions

cART-treated HIV-1-infected patients with lipodystrophy have a systemic overproduction of FABP-4, which is closely linked to insulin resistance and inflammatory markers in subcutaneous adipose tissue.

Keywords: dyslipidaemia, FABP-4, HIV-1 infection, insulin resistance, lipodystrophy, subcutaneous adipose tissue

Accepted 22 October 2010

Introduction

The widespread use of combination antiretroviral therapy (cART) has resulted in considerable success being achieved in improving mortality and morbidity outcomes in HIV-1-infected patients. Unfortunately, cART is associated with severe side effects, such as lipodystrophy, insulin resistance and a proatherogenic lipid profile, which may in time lead to increased cardiovascular morbidity [1–3]. Several adipokines involved in the inflammatory process related to insulin resistance and cardiovascular risk factors have been investigated previously in HIV-1-infected patients. A relationship between elevated inflammatory activity and adipose tissue changes has been proposed [4]. Despite their lower body fat content, an inflammatory profile similar to that found in insulin-resistant obese patients has been observed in HIV-1-infected patients with lipodystrophy [5]. In patients with lipodystrophy, higher levels of tumour necrosis factor (TNF)-α, interleukin-6 (IL-6) and IL-18 have been reported in both systemic and adipose tissue expression [6].

Recently, a newly discovered adipokine, fatty acid binding protein 4 (FABP-4; also called aP2), has emerged as an important mediator in the cross-talk between adipocytes and macrophages in adipose tissue. It belongs to the family of fatty acid binding proteins (FABPs) which are a group of molecules that co-ordinate lipid responses in cells and are also connected to metabolic and inflammatory pathways. FABPs are lipid chaperones that bind fatty acid ligands with high affinity and have functions in intracellular fatty acid trafficking, regulation of lipid metabolism, and modulation of gene expression [7,8]. FABP-4 is abundantly expressed in mature adipocytes and activated macrophages [9,10]. FABP-4-deficient mice exhibit higher insulin-stimulated glucose uptake and their adipocytes have a reduced efficiency of lipolysis, both *in vivo* and *in vitro*. Furthermore, studies of FABP-4 gene variants suggest that FABP-4 may have effects on plasma lipid levels and insulin sensitivity, and play a role in coronary heart disease [9,10]. All these data suggest that FABP-4 could be a potential target for the treatment of metabolic diseases.

Although it was once thought to be a purely intracellular protein, recent studies have shown FABP-4 to be present at high levels in human serum [11]. In cross-sectional analyses, circulating FABP-4 has been closely associated with obesity and metabolic syndrome, and in prospective studies FABP-4 levels predicted the development of metabolic syndrome and type 2 diabetes [11].

Data for HIV-1-infected patients are scarce. A recent study that included HIV-1-infected patients with metabolic syndrome and lipodystrophy showed that these patients had higher circulating levels of FABP-4 than those without metabolic syndrome or lipodystrophy, although the relationship with insulin resistance and other well-known inflammatory and adipocyte-related cytokines was not explored [12]. Considering that FABP-4 seems to be a key element in adipocyte differentiation, and that it has been postulated as a possible marker of fat distribution in mammals [13], we have hypothesized that FABP-4 may be involved in cART-related lipodystrophy syndrome and its associated metabolic disturbances in HIV-1-infected patients. We have therefore analysed the circulating levels of FABP-4 in an HIV-1-infected cohort including patients with and without lipodystrophy.

Materials and methods

Design, setting and participants

A multicentre cross-sectional case–control study was carried out. A total of 467 individuals were included in the study, all of whom were Caucasian adults, with 282 being HIV-1-infected and 185 uninfected. The HIV-1-infected patients were recruited from a prospectively collected cohort of 1700 HIV-1-infected individuals who had been receiving stable cART for at least 1 year. cART was defined as the combination of two nucleoside reverse transcriptase inhibitors (NRTIs) plus either a nonnucleoside reverse transcriptase inhibitor (NNRTI) or one or more protease inhibitors (PIs).

Regarding the HIV-infected patients, we recruited all patients with moderate or severe lipodystrophy (LD +), which was assessed clinically [14,15] ($n = 132$), and a randomly selected group of patients without lipodystrophy (LD − ; $n = 150$) whose age (± 5 years), gender, and duration of exposure to cART (± 3 months) were

comparable to those of the patients with lipodystrophy. The sample size was calculated to achieve a difference of FABP-4 levels greater than 6 ng/mL between groups that resulted in a confidence level of 95% and statistical power of 80%. The control group consisted of uninfected healthy subjects matched with patients for age and gender. The patients were followed up at the HIV-1 out-patient clinics of the three participating hospitals (Joan XXIII University Hospital of Tarragona, Santa Creu i Sant Pau Hospital, Barcelona and Sant Joan University Hospital, Reus). Inclusion criteria were age >18 years, presence of HIV-1 infection, stable cART regimen for at least 1 year and presence or absence of lipodystrophy according to clinical assessment (see below for categorization criteria). The presence of cachexia, active opportunistic infections, current inflammatory diseases or conditions, consumption of drugs with known metabolic effects such as steroids (topical, inhaled or systemic), antidiabetic or hypolipidaemic drugs and hormones, and plasma C reactive protein >1 mg/dL were considered as exclusion criteria for both patients and controls. All patients provided informed consent and the local ethics committees approved the study.

Clinical assessment of lipodystrophy

All HIV-1-infected patients were given a complete physical examination to assess the presence, type (lipoatrophy, lipohypertrophy or mixed) and degree (slight, moderate or severe) of lipodystrophy. Waist and hip circumference, height, weight and body mass index (BMI) were measured. The presence of lipodystrophy was defined as changes in body fat composition that were substantial enough to be recognized by both the patient and the attending physician. Criteria for lipoatrophy were one or more of the following: loss of fat from the face, arms and legs, prominent veins in the arms and legs, and loss of fat from the buttocks. Lipohypertrophy was defined as the presence of one or more of the following: an increase in abdominal perimeter, breast and/or neck fat deposition. We defined mixed lipodystrophy as occurring when at least one characteristic of lipoatrophy and one of lipohypertrophy were concomitantly present in a given patient. Lipodystrophy was categorized in accordance with the scale proposed by Carr *et al.* [1]: non-existent (0), slight (1), moderate (2) and severe (3). Doubtful cases were excluded. Lipodystrophy was evaluated according to this categorization in the face, arms, legs, buttocks, abdomen, neck and breasts. The sum of the values corresponding to each corporal zone indicated the degree of lipodystrophy: nonexistent (0), slight (1–6), moderate (7–12) and severe (13–18). In this study we included only moderate and severe cases in order to avoid an overlap between the LD+ and LD− subsets. The LD+ group comprised 26 patients with pure lipoatrophy and 106 patients with the mixed type. No cases of pure lipohypertrophy were recorded. With respect to severity, 109 had moderate and 23 had severe lipodystrophy.

Laboratory measurements

Collection of blood samples

After an overnight fast, 20 mL of blood obtained from a peripheral vein was collected in Vacutainer™ (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) ethylenediaminetetraacetic acid (EDTA) tubes. Five millilitres of whole blood was used to determine the CD4 T-cell count. Five hundred microlitres was used for DNA isolation with a MagNa Pure LC Instrument (Roche Diagnostics, Basel, Switzerland). Plasma and serum were obtained by centrifugation at 3500 *g* for 15 min at 4 °C and stored at −80 °C until use.

HIV-1-related data

HIV-1 infection and plasma HIV-1 viral load were assessed as described elsewhere [14]. The CD4 T-cell count was determined using a flow cytometer FAC Scan (Becton Dickinson Immunocytometry Systems). Data acquired were analysed using the MULTISET program (Becton Dickinson Immunocytometry Systems).

Blood chemistry

Plasma glucose, total cholesterol, HDL cholesterol and triglycerides were determined in an ADVIA 1200 (Siemens AG, Munich, Germany) auto-analyser using standard enzyme methods. Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald formula [16]. Fasting plasma insulin was measured using a specific immunoradiometric assay (Medgenix Diagnostics, Fleunes, Belgium) in which proinsulin did not cross-react. The intra- and inter-assay coefficients of variation (CVs) were 6% and 7%, respectively. The homeostasis model assessment of insulin resistance (HOMA-IR) as a marker for insulin resistance was calculated according to the formula [fasting glucose (in millimoles per litre) × fasting insulin (in microunits per millilitre)/22.5] [17].

Plasma levels of cytokines and adipokines

Soluble tumour necrosis factor receptor 1 (sTNF-R1) and sTNF-R2 were assessed as previously described [18]. Adiponectin levels were measured using a standardized radioimmunoassay kit from Linco Research (Linco Research Inc., St. Charles, MO, USA). The kit has a sensitivity of 1 ng/mL. The intra- and inter-assay CVs were 8% and 12%, respectively. Plasma FABP-4 was measured using the

Human Adipocyte FABP ELISA (BioVendor Laboratory Medicine, Palackeho, Czech Republic). The sensitivity was 0.1 ng/mL. The intra- and inter-assay CVs were 5.2% and 3.8%, respectively. The leptin concentration in plasma was determined with a Human Leptin ELISA kit (Assaypro, St Charles, MO, USA); the lowest detectable level was 0.15 pg/mL with an intra-assay CV of 4.0% and an inter-assay CV of 7.7%. The IL-18 concentration in plasma was determined with a Human IL-18 ELISA kit (MBL International Corporation, Woburn, MA, USA); the lowest detectable level was 12.5 pg/mL. The intra-assay CV was 7.2% and the inter-assay CV was 7.5%. Plasma IL-6 levels were measured using the commercial kit Human IL-6 Quantikine HS High Sensitivity (R&D Systems, Lille, France).

Adipose tissue samples

Samples of subcutaneous adipose tissue (SAT) were obtained from subcutaneous abdominal depots by a small surgical biopsy, under local anaesthesia with mepivacaine. Twenty-five HIV-1-infected patients with lipodystrophy (LD+), and 13 HIV-1-infected patients without lipodystrophy (LD−) were biopsied. All patients had fasted overnight. One to four grams of SAT was removed from each biopsy and immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was extracted from 400–500 mg of frozen SAT using the RNeasy Lipid Tissue Midi Kit (Qiagen Science, Valencia, CA, USA) according to the manufacturer's instructions. One microgram of RNA was retrotranscribed to cDNA using the Reverse Transcription System (Promega Corporation, Madison, WI, USA) in a final volume of 20 μL. The following primers were used in the real-time quantitative polymerase chain reaction: 5-gagcactgaaagcatgatcc-3 and 5-gctggttat ctctcagctcca-3 for *TNF-α*, 5-tctgtgcctgctgctcatag-3 and 5-cagatctccttggccacaat-3 for monocyte chemoattractant protein-1 (*MCP-1*); 5-ggaaactcaagcctgcactc-3 and 5-ggatg aagtcgtgttggaga-3 for *TNF-R1*; 5-tgccgctgtgtaggaaagaa-3 and 5-gctcacaaggttctggcg-3 for *TNF-R2*; 5-atgaggctgg ctgtgctt-3 and 5-gtggttttgtggctcttggt-3 for *CD68* and 5-ctatggagttcatgcttgtg-3 and 5-gtactgacatttattt-3 for peroxisome proliferator activated receptor gamma (*PPAR-γ*). The housekeeping genes used to normalize gene expression were: β-actin, 5-ggacttcgagcaagagatgg-3 and 5-agcactg tgttggcgtacag-3, and cyclophilin A (*CYPA*), 5-caaatgctgg acccaacac-3 and 5-gcctccacaatattcatgccttctt-3.

Statistical analyses

All statistical analyses were performed using the SPSS 13.0 software (SPSS, Chicago, IL, USA). We performed the one-sample Kolmogorov–Smirnov test to verify the normal distribution of the quantitative variables. Normally distributed data are expressed as the mean ± standard deviation (SD), whereas variables with a skewed distribution are represented as the median (interquartile range). Categorical variables are reported as number (percentage). Student's *t*-test was used to compare the mean values of continuous variables normally distributed between independent groups. For variables with skewed distributions, we used the Kruskal–Wallis test. To analyse the differences in nominal variables between groups, we used the χ^2 test. Spearman's correlation coefficient was used to analyse the bivariate correlation between FABP-4 and metabolic parameters. A stepwise multiple regression analysis including several metabolic and clinical variables known to be associated with insulin sensitivity was performed to determine whether FABP-4 was independently associated with HOMA-IR. A logistic regression analysis was performed to determine the OR of FABP for the presence of lipodystrophy after adjustment for age, sex and BMI. FABP-4 levels were also grouped into tertiles and a logistic regression analysis was performed to determine the OR for the presence of lipodystrophy in subjects in the higher FABP-4 tertiles compared with those in the lowest tertile. For all comparisons, a P value <0.05 was considered significant.

Results

Baseline characteristics of the subjects

Whole group

The main clinical and metabolic characteristics of healthy controls and HIV-1-infected patients are shown in Table 1. Uninfected subjects had a higher mean BMI than HIV-1-infected patients ($P<0.001$). As expected, levels of inflammatory parameters (sTNF-R2, IL-6 and IL-18; $P<0.001$ for all) were higher in HIV-1-infected patients. Leptin levels were significantly lower in HIV-1-infected patients ($P<0.001$). In contrast, sTNF-R1 and adiponectin did not show significant differences between the groups.

HIV-1-infected subjects

Table 2 shows the main characteristics of the HIV-1-infected cohort, categorized according to the presence or absence of lipodystrophy. As expected, the group with lipodystrophy (LD+) had significantly higher mean BMI and waist/hip circumference ratio. They also had more advanced disease, as defined by the Centers for Disease Control and Prevention (CDC) classification, and a greater CD4 T-cell increase attributable to cART, compared with those without lipodystrophy (LD−). Moreover, LD+ patients had received a higher number of PIs and NRTIs and had had more prolonged exposure to NRTIs (Table 2),

Table 1 Clinical, metabolic and adipokine characteristics of healthy controls and HIV-1-infected patients

	Healthy controls ($n = 185$)	HIV-1-infected patients ($n = 282$)	*P*
Age (years)	40.00 (31.00–50.25)	42.00 (36.00–45.00)	0.805
Gender [*n* (%) male]	122 (65.9)	185 (65.6)	0.921
Waist circumference (cm)	90.00 (80–95)	88.00 (80–93)	0.378
BMI (kg/m^2)	25.75 ± 4.13	23.18 ± 2.99	<0.001
Plasma insulin (μU/mL)	5.95 (3.75–13.49)	6.96 (4.03–14.65)	0.435
Plasma adiponectin (μg/mL)	11.815 (7.635–19.223)	10.713 (4.906–19.032)	0.101
Plasma sTNF-R1 (ng/mL)	1.89 (1.50–2.40)	1.96 (1.57–2.45)	0.406
Plasma sTNF-R2 (ng/mL)	3.27 (2.80–3.80)	5.56 (4.34–7.62)	<0.001
Plasma leptin (ng/mL)	8.56 (4.94–14.58)	4.16 (2.33–7.39)	<0.001
Plasma IL-6 (pg/mL)	1.11 (0.65–1.77)	1.66 (1.18–2.73)	<0.001
Plasma IL-18 (pg/mL)	185.68 (147.24–233.61)	298.93 (224.50–409.15)	<0.001
Plasma FABP-4 (ng/mL)	21.21 (13.97–41.42)	24.63 (13.27–38.99)	0.221

Data are expressed as mean ± standard deviation or median (interquartile range).
BMI, body mass index; FABP-4, fatty acid binding protein 4; IL-18, interleukin-18; sTNF-R1, soluble tumour necrosis factor receptor 1; sTNF-R2, soluble tumour necrosis factor receptor 2.

particularly stavudine (d4T). No differences in FABP-4 levels were observed according to the antiretroviral drugs received.

With respect to the metabolic and inflammatory parameters, LD + patients had higher mean insulin ($P<0.001$), triglyceride ($P<0.001$), total cholesterol ($P=0.005$) and LDL cholesterol ($P=0.038$) plasma levels, but lower mean HDL cholesterol levels ($P<0.001$). The HOMA-IR index was also significantly higher in the LD + group ($P<0.001$). Circulating levels of sTNF-R1, sTNF-R2, IL-6 and IL-18 were similar in the two HIV-1-infected groups. Patients with lipodystrophy had significantly lower adiponectin ($P<0.001$) and significantly higher leptin ($P=0.008$) plasma levels compared with the nonlipodystrophy subset. Before considering patients with lipodystrophy as a whole, we investigated differences in inflammatory and metabolic parameters between patients with moderate and severe lipodystrophy, and also between patients with the mixed type of lipodystrophy and those with lipoatrophy. No differences were found (data not shown).

Plasma FABP-4 levels

HIV-1-infected patients had similar plasma FABP-4 levels to uninfected controls (Table 1). However, among infected patients, plasma FABP-4 levels were significantly higher in those with lipodystrophy than in those without lipodystrophy ($P=0.012$) (Table 2). FABP-4 levels were similar between patients with different types and severities of lipodystrophy, and among those with different levels of exposure to antiretroviral drugs.

Odds ratios for lipodystrophy for FABP-4 subsets

In multiple regression analysis, after adjustment for age, BMI and sex, high FABP-4 levels were significantly associated with lipodystrophy [odds ratio (OR) 1.016; 95% confidence interval (CI) 1.01-1.027; $P=0.004$]. To determine the OR for the presence of lipodystrophy in patients with higher FABP-4 levels, we used tertiles to categorize the FABP-4 level, and carried out a multiple logistic regression analysis (Table 3). Patients in the highest FABP-4 tertile had a higher OR for the presence of lipodystrophy than those in the middle tertile. The OR for those in the highest tertile remained significant after adjustment for sex, BMI and age.

Relationship between plasma FABP-4 level and metabolic and inflammatory parameters

In the whole HIV-1-infected cohort, bivariate correlation analyses showed significant correlations between circulating FABP-4 level and some clinical and metabolic traits. Correlations were positive with BMI ($P<0.001$), insulin ($P<0.001$), HOMA-IR ($P<0.001$), total cholesterol ($P=0.013$), LDL cholesterol ($P=0.040$) and triglycerides ($P<0.001$), and negative with HDL cholesterol ($P=0.002$) (Table 4). Regarding immunological and inflammatory parameters, significant positive correlations were observed between plasma FABP-4 level and sTNF-R1 ($P<0.001$), leptin ($P<0.001$) and IL-18 ($P=0.034$) plasma levels (Table 4), while a negative correlation was observed with adiponectin ($P=0.006$).

When we analysed data for HIV-1-infected patients separately in the LD + and LD − groups, both subsets showed a positive association between FABP-4 plasma level and BMI, fasting insulin and HOMA-IR index (Table 4). In contrast, triglycerides were only positively correlated with FABP-4 in LD + patients ($P=0.035$). Regarding immunological and inflammatory parameters, only leptin was positively correlated with plasma FABP-4 level in both the LD + and LD − groups. Positive correlations between plasma FABP-4 level and sTNF-R1 ($P=0.039$), sTNF-R2 ($P<0.001$) and IL-18 ($P=0.029$) were also found in the LD + subset (Table 4).

Table 2 Clinical and metabolic characteristics of HIV-1-infected subjects categorized according to the presence of lipodystrophy

Variable	LD − patients ($n = 150$)	LD + patients ($n = 132$)	*P*
Age (years)	40 (36–44)	40 (36–47)	0.206
Gender (% male)	67.9	60.0	0.168
Body mass index (kg/m^2)	22.33 (20.59–24.50)	23.45 (21.64–25.52)	0.01
Waist:hip circumference ratio	0.89 ± 0.09	0.94 ± 0.08	<0.001
Glucose (mmol/L)	5.10 (4.60–5.50)	5.20 (4.70–5.79)	0.129
Insulin (μU/mL)	5.18 (3.61–11.02)	8.61 (5.25–19.27)	<0.001
HOMA-IR	1.27 (0.83–2.41)	1.97 (1.18–4.36)	<0.001
Triglycerides (mmol/L)	1.51 (0.90–2.12)	2.25 (1.46–3.80)	<0.001
Total cholesterol (mmol/L)	4.94 ± 1.18	5.47 ± 1.42	0.005
LDL cholesterol (mmol/L)	2.85 ± 1.00	3.29 ± 1.16	0.038
HDL cholesterol (mmol/L)	1.20 (0.99–1.47)	1.00 (0.82–1.30)	<0.001
cART duration (months)	60.82 ± 30	54.11 ± 24.39	0.145
Exposed to NRTIs before cART (%)	34	53	0.059
Ever exposed to NRTIs (%)	94	100	0.003
Cumulative time on NRTIs (months)	99.05 ± 61.25	122.09 ± 52.07	0.001
Cumulative time on d4T (months)	14.18 ± 20.37	34.59 ± 22.00	<0.001
Cumulative time on ZDV (months)	33.0 ± 29.9	26.2 ± 26.0	0.04
Cumulative time on PIs (months)	30.94 ± 29.62	33.28 ± 24.68	0.494
Ever exposed to PIs (%)	74.5	84.0	0.036
Cumulative time on NNRTIs (months)	14.40 ± 17.16	17.23 ± 16.82	0.181
Ever exposed to NNRTIs (%)	68.2	72.0	0.385
CD4 count (cells/μL)			
Nadir	329 ± 270	265 ± 207	0.040
Current	529 ± 316	577 ± 327	0.200
HIV RNA load			
Pre-cART ($\log_{10}$ copies/mL)	4.6 ± 1.1	4.5 ± 1.4	0.500
Post-cART ($\log_{10}$ copies/mL)	2.4 ± 1.4	2.2 ± 0.8	0.100
AIDS (categories A3, B3 and C) (%)	35.0	30.3	0.020
Plasma sTNF-R1 (ng/mL)	1.96 (1.56–2.37)	1.96 (1.57–2.58)	0.552
Plasma sTNF-R2 (ng/mL)	5.74 (4.29–7.76)	5.45 (4.29–7.57)	0.983
Plasma IL-6 (pg/mL)	1.65 (1.09–2.78)	1.81 (1.21–2.63)	0.898
Plasma IL-18 (pg/mL)	296.35 (211.00–412.08)	300.24 (235.13–407.74)	0.447
Plasma adiponectin (μg/mL)	14.439 (7.959–20.783)	5.719 (3.045–15.139)	<0.001
Plasma leptin (ng/mL)	4.58 (2.52–7.79)	4.80 (2.09–6.96)	0.008
Plasma FABP-4 (ng/mL)	18.48 (12.68–30.51)	26.00 (13.96–46.62)	0.012

Data are expressed as mean ± standard deviation or median (interquartile range).
Use of other antiretroviral drugs (didanosine, zalcitabine, lamivudine, abacavir, tenofovir, nevirapine, saquinavir, indinavir, ritonavir, nelfinavir, amprenavir, lopinavir and atazanavir) did not differ significantly between groups.
BMI, body mass index; cART, combination antiretroviral therapy; d4T, stavudine; FABP-4, fatty acid binding protein 4; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IL-18, interleukin-18; LD −, HIV-1-infected patients without lipodystrophy; LD +, HIV-1-infected patients with lipodystrophy; LDL, low-density lipoprotein; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; sTNF-R1, soluble tumour necrosis factor receptor 1; sTNF-R2, soluble tumour necrosis factor receptor 2; ZDV, zidovudine.

To investigate whether the degree of insulin resistance was independently associated with FABP-4 level, we developed a stepwise multiple linear regression analysis including HOMA-IR as a dependent variable and serum FABP-4 and other clinical and metabolic variables known to be related to insulin resistance as covariates. FABP-4 was one of the five variables included in the model ($P = 0.004$) (Table 5). The variables excluded ($P > 0.05$) were sex, BMI, leptin, HDL cholesterol, LDL cholesterol, total cholesterol, triglycerides and adiponectin.

Relationship between FABP-4 plasma level and SAT gene expression

SAT biopsies from 38 HIV-1-infected patients (25 LD + and 13 LD −) were available (Tables 6 and 7). The use of NRTIs or NNRTIs did not affect the genetic expression profile. The expression of *TNF-R1* and *MCP-1* was lower in patients on PI drugs, but no differences in the genetic expression profile according to the antiretroviral agent used were found when the LD + and LD − groups were considered separately (data not shown). LD + patients showed higher levels of *CD68* ($P < 0.001$), *TNF-R1* ($P = 0.029$) and *TNF-R2* ($P = 0.044$) than LD − patients. Moreover, *CD68* and *MCP-1* gene expression showed a positive correlation with circulating FABP-4 level ($P = 0.022$ and $P = 0.046$, respectively) while *PPAR-γ* expression showed a negative correlation with circulating FABP-4 level in the HIV-1-infected group as a whole. When analyses of these relationships were carried out separately in the LD + and LD − groups, FABP-4 remained

Table 3 Odds ratios (ORs) for the presence of lipodystrophy according to fatty acid binding protein 4 (FABP-4) level categorized in tertiles

	OR (95% CI)		
	Tertile 1 (3.50–15.53)	**Tertile 2 (15.54–30.88)**	**Tertile 3 (30.89–203.77)**
Model 1	1	1 (0.541–1.848)	2.917 (1.581–5.380)
Model 2	1	0.838 (0.435–1.616)	2.281 (1.163–4.475)

Model 1 is unadjusted; model 2 is model 1, plus correction for age, sex and body mass index. Values in brackets indicate FABP-4 concentration.
CI, confidence interval.

Table 4 Correlations between fatty acid binding protein 4 (FABP-4) plasma level and clinical and metabolic parameters in HIV-1-infected patients

	All HIV-infected patients ($n = 282$)		**LD + patients ($n = 132$)**		**LD − patients ($n = 150$)**	
	r	***P***	***r***	***P***	***r***	***P***
Age	0.115	0.070	0.146	0.119	0.068.	0.436
BMI	0.284	<0.001	0.321	<0.001	0.189	0.044
Insulin	0.341	<0.001	0.232	0.014	0.388	<0.001
HOMA-IR	0.362	<0.001	0.282	0.002	0.371	<0.001
Cholesterol	0.156	0.013	−0.012	0.892	0.192	0.020
HDL cholesterol	−0.197	0.002	−0.121	0.162	−0.206	0.027
LDL cholesterol	0.134	0.040	0.111	0.269	0.111	0.206
Triglycerides	0.219	<0.001	0.198	0.035	0.155	0.073
cART duration (months)	−0.006	0.948	0.005	0.965	0.009	0.945
Time on PIs (months)	−0.036	0.566	−0.086	0.354	−0.011	0.900
Time on NRTIs (months)	0.083	0.178	0.032	0.731	0.065	0.441
Time on NNRTIs	0.173	0.005	0.078	0.401	0.246	0.003
Plasma sTNF-R1	0.232	<0.001	0.312	0.039	0.164	0.059
Plasma sTNF-R2	0.123	0.052	0.340	<0.001	−0.063	0.467
Plasma adiponectin	−0.170	0.006	−0.124	0.181	−0.092	0.271
Plasma leptin	0.306	<0.001	0.319	0.001	0.382	<0.001
Plasma IL-6	0.105	0.088	0.164	0.075	0.063	0.611
Plasma IL-18	0.135	0.034	0.204	0.029	0.062	0.480

BMI, body mass index; cART, combination antiretroviral therapy; HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; IL-18, interleukin-18, LDL, low-density lipoprotein; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; sTNF-R1, soluble tumour necrosis factor receptor 1; sTNF-R2, soluble tumour necrosis factor receptor 2.

positively correlated only with *CD68* expression in the LD + group ($P = 0.031$). No other significant correlations with FABP-4 plasma level were observed.

Discussion

This study provides some meaningful insights into the involvement of FABP-4 in cART-related lipodystrophy in HIV-1-infected patients. We observed systemic overproduction of FABP-4 in cART-treated HIV-1-infected patients with lipodystrophy and found that those with a plasma FABP-4 level in the highest tertile had a higher prevalence of lipodystrophy. Furthermore, we found that FABP-4 was one of the major determinants of the degree of insulin resistance in HIV-1-infected patients, and this association

Table 5 Stepwise multiple linear regression analysis of the relationship between the homeostasis model assessment of insulin resistance (HOMA-IR) index* and the fatty acid binding protein 4 (FABP-4) concentration and clinical, inflammatory and metabolic parameters

Independent variables	**Beta**	***t***	***P***
Waist:hip ratio	0.249	3.142	0.002
Plasma FABP-4*	0.236	2.903	0.004
Plasma sTNF-R1	−0.231	−2.936	0.004
Lipodystrophy	0.190	2.412	0.017
Plasma IL-18	0.150	1.981	0.050

r^2: 0.257.
IL-18, interleukin-18; sTNF-R1, soluble tumour necrosis factor receptor 1.
*Log-transformed.

Table 6 Subcutaneous adipose tissue gene expression of some inflammatory markers

mRNA expression	**LD + patients**	**LD − patients**	***P***
CD68	0.154 ± 0.076	0.080 ± 0.24	<0.001
TNF-α	0.145 (0.099–0.221)	0.112 (0.058–0.201)	0.533
*TNF-R1**	1.776 ± 1.166	2.731 ± 1.347	0.029
*TNF-R2**	1.740 ± 1.204	2.701 ± 1.575	0.044
MCP-1†	2.189 (1.132–3.195)	1.377 (1.098–1.886)	0.311
PPAR-γ	0.462 (0.416–0.764)	0.668 (0.450–0.761)	0.270

Data are expressed as mean ± standard deviation for normally distributed variables or median (interquartile range) for variables with a skewed distribution.
LD, lipodystrophy; *MCP-1*, monocyte chemoattractant protein 1; *PPAR-γ*, peroxisome proliferator activated receptor gamma; *sTNF-R1*, soluble tumour necrosis factor receptor 1; *sTNF-R2*, soluble tumour necrosis factor receptor 2.
*Multiplied by 10^{-3}.
†Multiplied by 10^{-2}.

Table 7 Correlations between fatty acid binding protein 4 (FABP-4) plasma level and subcutaneous adipose tissue (SAT) mRNA expression

	All HIV-1-infected patients		**LD + patients**		**LD − patients**	
	r	***P***	***r***	***P***	***r***	***P***
CD68	0.423	0.022	0.525	0.031	0.098	0.762
TNF-α	0.163	0.397	0.279	0.248	0.098	0.762
TNF-R1	−0.042	0.827	−0.373	0.141	0.346	0.247
TNF-R2	−0.306	0.100	−0.309	0.228	0.286	0.334
MCP-1	0.367	0.046	0.390	0.122	0.027	0.929
PPAR-γ	−0.233	0.227	−0.522	0.032	−0.236	0.437

MCP-1, macrophage chemoattractant protein 1; *PPAR-γ*, peroxisome proliferator activated receptor gamma; *TNF-α*, tumour necrosis factor alpha; *TNF-R1*, tumour necrosis factor alpha receptor 1; *TNF-R2*, tumour necrosis factor alpha receptor 2.

was independent of body fat distribution. We also observed a close relationship between FABP-4 and inflammatory markers both in plasma and in SAT.

The biological role of circulating FABP-4 is not well understood, but the association observed between serum FABP-4 level and the development of atherosclerosis, metabolic syndrome and type 2 diabetes suggests that plasma FABP-4 levels may parallel its tissue expression and activity. In our HIV-1-infected cohort, FABP-4 levels were similar to those observed in the control group, despite the difference between the groups in BMI, suggesting that other inflammatory factors could play a role in the regulation of this protein in this population. The observed increase in circulating FABP-4 levels in LD + HIV-1-infected subjects is consistent with some previous reports in which this protein was evaluated in the context of HIV-1 infection. Similar to our results, Coll and colleagues reported that the level of circulating FABP-4 was higher in HIV-1-infected patients with lipodystrophy compared with nonlipodystrophic subjects, and was closely correlated with BMI and insulin level [12]. However, in that study no measurements of inflammatory parameters or insulin resistance were made. In our cohort, findings for HIV-1-infected patients were similar to those for the uninfected group, and the plasma FABP-4 level was clearly associated with BMI, HOMA-IR index, inflammatory markers and dyslipidaemia. Regarding insulin sensitivity, in an analysis of the variables associated with the HOMA-IR index, we found that FABP-4 level was one of the variables most strongly associated with insulin sensitivity, irrespective of the presence or absence of lipodystrophy. Interestingly, significant associations between FABP-4 plasma level and inflammatory markers expressed in adipose tissue were found mainly in LD + patients. Both sTNF-R1 and sTNF-R2, surrogate markers for TNF-α activity, were higher in LD + patients. These findings are in agreement with previous reports in which increased levels of IL-6 were found in this subset of patients [19].

As FABP-4 has been suggested to be an adipocytokine involved in the cross-talk between adipocytes and macrophages, we investigated whether there was any relationship between FABP-4 serum level and the expression of markers of inflammation and macrophage infiltration in SAT biopsies obtained from patients with and without lipodystrophy. Up-regulation of *CD68* gene expression, a macrophage marker, was found in LD + patients, indicating an inflammatory local environment in SAT. Interestingly, *CD68* expression was found to be closely associated with the level of circulating FABP-4 only in LD + HIV-1-infected patients. Taken together, these results indicate a more aggressive inflammatory pattern both at the paracrine and at the systemic level in the context of HIV-1-associated lipodystrophy. It is difficult to extrapolate the local data obtained in adipose tissue to the systemic inflammatory profile, but this relationship is particularly relevant in LD + patients. In agreement with previous reports [12], in our HIV-1-infected cohort, FABP-4 was found to be closely associated with lipodystrophy, independently of BMI, sex and age. Although we cannot discount the possibility that exposure to PIs and NRTIs could contribute to the high FABP-4 levels observed in the LD + group, results of previous experiments on the effects of PIs and NRTIs indicate that they block adipocyte differentiation. It was found that PIs interfere with adipocyte differentiation whereas NRTIs decrease *PPAR*-γ expression in adipose tissue. Both PPAR-γ and FABP-4 mRNA expression in adipose tissue increased in both NRTI-exposed and non-exposed after rosiglitazone treatment [20]. These observations argue against a direct effect of these treatments on FABP-4 expression via PPAR-γ in HIV-1-infected LD + patients, or at least against an effect with significant systemic repercussions for circulating plasma levels. Consistent with this conclusion, we observed that LD + patients were more frequently treated with PIs and NRTIs than LD − subjects, but FABP-4 levels were similar when the groups were compared according to NRTI and PI treatment (data not shown). In contrast, similar proportions of patients were treated with NNRTIs in the two groups, but in both cases FABP-4 levels were higher in patients treated with NNRTIs than in other patients in the same group. The absence of relationship of any of the antiretroviral drugs with FABP-4 levels in the Coll *et al*. study also argues against an important effect of cART on FABP-4 levels [12].

FABP-4 plasma levels were closely related to adipose tissue expression of *CD68* and *MCP-1* in HIV-1-infected patients, but when the LD + and LD − groups were considered separately the association with *CD68* expression was found only in the LD + subset of patients, suggesting that intense macrophage infiltration is associated with lipodystrophy.

This study has limitations. First, the cross-sectional nature of our study design (and hence the single measurement of FABP-4 in the study) means that our results provide information about associations but not causality. Secondly, we defined lipodystrophy clinically and cannot discount the possibility that some patients in the LD − group could have had minor subclinical changes that were not clinically detectable. However, we believe that this is unlikely because our cohort comprised patients with extreme phenotypes. Finally, we do not have the FABP-4 mRNA expression levels in SAT and this may have limited the interpretation of data on inflammatory markers in this tissue. Investigation of FABP-4 expression in adipose tissue from patients with lipodystrophy may prove

beneficial in the development of possible therapeutic options.

FABP-4 has been suggested as a potential therapeutic target for patients with type 2 diabetes, obesity and atherosclerosis [21]. It has been observed that patients with the genetic variant of the *FABP-4* gene (T-87C) associated with reduced transcriptional activity of the gene and diminished *FABP-4* expression in adipose tissue have lower triglyceride levels and a reduced risk of developing obesity and type 2 diabetes [21]. Recently, investigation of pharmacological agents that inhibit FABP-4 function in experimental models has yielded promising results [10], but further studies are needed to determine whether such agents may be of benefit in LD + patients.

In summary, our data suggest involvement of the FABP-4 system in cART-related lipodystrophy in HIV-1-infected patients who have increased systemic FABP-4 production, and that this increased FABP-4 production is probably related to macrophage adipose tissue gene expression. A close relationship between insulin resistance and FABP-4 level was found in the HIV-1-infected cohort, suggesting that FABP-4 may play a role in the carbohydrate metabolism disturbances observed in these patients. We propose that FABP-4 may influence both systemic and local inflammatory responses in HIV-1-infected patients with cART-associated lipodystrophy.

Acknowledgements

This work was partially financed by grants from the Fondo de Investigacion Sanitaria (07/0976, 08/1032, 08/1195, 08/1715 and 10/2635); Fondos Europeos para el Desarrollo Regional (FEDER); Fundación para la Investigación y Prevención del Sida en España (FIPSE 06/36572, 06/36610 and 36-0998-10); Ministerio de Ciencia e Innovación (SAF2008-02278); Programa de suport als Grups de Recerca AGAUR (2009 SGR 284, 959, 1061 and 1257); Red de Investigación en Sida (RIS, RD06/006/0022 and RD06/0006/1004); and the Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Spain. XE is supported by a fellowship from the JdlC programme and grant JDCI20071020. CIBERdem and CIBERobn are ISCIII projects. The comments and critiques of the anonymous reviewers helped us to improve the manuscript and are greatly appreciated.

Appendix

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Genetic and Functional Mitochondrial Assessment of HIV-Infected Patients Developing HAART-Related Hyperlactatemia

Revista: Journal of Acquired Immune Deficiency Syndrome. 2009 Dec 1; 52(4):443-51.

PMID: 19779356

Título: Valoración genética y de función mitocondrial de pacientes infectados por HIV-1 que han desarrollado hiperlactatemia relacionada a tratamiento HAART

El daño mitocondrial provocado por los medicamentos de la terapia HAART, especialmente los análogos de nucleósidos, interfieren en la replicación del DNA mitocondrial. Esto ha sido descrito como una de las bases que provoca la hiperlactatemia relacionada con la terapia HAART, pero se han realizado escasos estudios para correlacionar las manifestaciones clínicas con las alteraciones mitocondriales. En este estudio se observó que los parámetros mitocondriales disminuían durante la hiperlactatemia y mejoraban al recuperarse el paciente. Los productos de replicación y transcripción mitocondrial se vieron reducidos, pero el descenso más marcado fue en el contenido de proteínas mitocondriales y las actividades de los complejos mitocondriales III y IV. Durante la hiperlactatemia los niveles de lactato correlacionaban con la función de los complejos mitocondriales III y IV. Tras la recuperación los niveles de parámetros mitocondriales recuperaron valores de individuos infectados por HIV-1 no hiperlactatémicos, que eran más bajos que los rangos de los pacientes naive e individuos sanos control. Se concluye que la hiperlactatemia relacionada con la terapia HAART se asocia con un impedimento mitocondrial generalizado que se revierte tras la recuperación del paciente. La bioquímica mitocondrial muestra una mejor correlación con los niveles de lactato que la genética mitocondrial sugiriendo que la función mitocondrial puede ser un mejor marcador del desarrollo de la hiperlactatemia que el contenido de DNA mitocondrial.

Genetic and Functional Mitochondrial Assessment of HIV-Infected Patients Developing HAART-Related Hyperlactatemia

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Background: Mitochondrial damage of HIV and antiretrovirals, especially nucleoside-analogue interference on mitochondrial DNA (mtDNA) replication, is reported to underlay highly active antiretroviral therapy (HAART)–related hyperlactatemia, but scarce approaches have been performed to correlate clinical manifestations and mitochondrial abnormalities.

Methods: We obtained lymphocytes and monocytes of 26 HIV-infected and treated patients who developed hyperlactatemia and after recovery, 28 nonhyperlactatemic HIV subjects on HAART, 31 naive individuals, and 20 uninfected controls. Mitochondrial replication and transcription analysis were performed by quantitative real-time PCR, mitochondrial translation quantification by western blot and mitochondrial enzymatic activities by spectrophotometry.

Results: Mitochondrial parameters decreased during hyperlactatemia and improved at recovery. Mitochondrial replication and transcription species were reduced ($P = 0.16$ and $P = 0.71$), but the most significant decay was observed on mitochondrial protein content ($P < 0.05$) and mitochondrial complexes III and IV activities ($P < 0.01$ and $P < 0.001$). During hyperlactatemia lactate level correlated complexes III and IV function ($P < 0.05$). After recovery mitochondrial parameters achieved values of nonhyperlactatemic HIV individuals, which were lower than ranges of naive subjects and uninfected controls.

Conclusions: HIV and HAART-related hyperlactatemia is associated with a general mitochondrial impairment which reverts after recovery. Mitochondrial biochemistry show a better correlation with lactate levels than mitochondrial genetics suggesting that mitochondrial function could be a better marker of hyperlactatemia development than mtDNA content.

Key Words: HIV, HAART, hyperlactatemia, mitochondria, mitochondrial toxicity, mitochondrial function/dysfunction

(*J Acquir Immune Defic Syndr* 2009;52:477–485)

INTRODUCTION

Lai et al[1] reported in 1991 for the first time a case of severe lactic acidosis and fulminant hepatic failure in an HIV-infected patient on didanosine (ddI) antiretroviral monotherapy. Since then, the introduction of the highly active antiretroviral therapy (HAART) has increased the risk of suffering from secondary effects and several additional reports have confirmed that patients who receive some nucleoside analogues reverse transcriptase inhibitors (NRTIs),

Received for publication April 7, 2009; accepted August 18, 2009.

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Preliminary data which represent only a small part of the information contained on the present manuscript have been presented as poster communication to the following meetings: (1) Congress: 4th IAS Conference on HIV Pathogenesis, Treatment and Prevention by Garrabou G, López S, Morén C, Rodriguez V, Milinkovic A, Martinez E, Riba J, Casademont J, Cardellach F, Gatell JM, Miró Ò. "Mitochondrial impairment in mononuclear cells of hyperlactatemic patients on HAART." Sydney (Australia), July 22–25, 2007. (2) At the same time it was co-submitted to the 9th International Workshop on Adverse Drug Reactions and Lipodystrophy in HIV by Garrabou G, López S, Morén C, Rodriguez V, Milinkovic A, Martinez E, Riba J, Casademont J, Cardellach F, Gatell JM, Miró Ò. Mitochondrial impairment in mononuclear cells of hyperlactatemic patients on HAART. Sydney (Australia), July 19–22, 2007.

Supported by Fundació la Marató de TV3 (02/0210 and 02/0631), Fundación para la Investigación y la Prevención del SIDA en España (FIPSE 36612/06 and 36572/06), Fondo de Investigación Sanitaria (FIS 0381/04, 1239/04 0229/08 and 1715/08), Red de Sida (RD 06/006), Suports a Grups de Recerca de la Generalitat de Catalunya (2009/SGR/1158) and CIBER de Enfermedades Raras and CIBER de Fisiopatología de la Obesidad y Nutrición (initiatives of the ISCIII). Dr. Òscar Miró has been depositary of a Research Intensification grant from ISCIII (Spain) during 2009.

Disclosure statement/conflict of interest: This work has not been supported by pharmaceutical funding which could have commercial interest on the present results. None of the authors has a financial or beneficial interest in the products or concepts mentioned in the present article or in competing products that might bias his/her judgment. None of them is in association with any organization that could pose a conflict of interest on the points described on the present manuscript.

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particularly ddI and stavudine (d4T), are at increased risk for developing hyperlactatemia and lactic acidosis,[2,3] a condition thought to be amongst the most serious adverse effects attributed to HIV and antiretroviral drugs.

Increased blood lactate levels may be associated with a multitude of accompanying and unspecific symptoms including fatigue, weakness, abdominal pain, weight loss, tachycardia, or dyspnea. The clinical presentation of hyperlactatemia is strikingly variable and the severity of symptoms usually shows a good positive correlation with plasma lactate levels. Cohort studies report that 15%–20% of persons/yr who receive a stable HAART regimen develop asymptomatic hyperlactatemia[3]; these patients show blood lactate levels usually between 2 and 5 mmol/L. Otherwise, 1% of patients/yr develop symptomatic hyperlactatemia, which is usually associated with plasma lactate levels above 5 mmol/L and one or more of the above mentioned manifestations.[3] Finally, 0.4–1‰ of patients on HAART/yr present lactic acidosis (blood pH imbalance), which is often associated with higher lactate levels and severe symptomatology, which can lead to death in up to 50% of cases through fulminant hepatic failure.[2,3] The prevalence and incidence of hyperlactatemic-related disorders is increasing in developing countries (because of growing access to antiretrovirals) and shows a trend towards reduction in the developed world because of physicians awareness and available routinary lactate measures, although unfortunately it is still a quite frequent and life-threatening event.

Mitochondrial toxicity of antiretroviral drugs, particularly NRTIs, has been postulated to be responsible for the etiopathogesis of many secondary effects of HAART,[4,5] including hyperlactatemia. Moreover, HIV itself could extend the mitochondrial adverse effects of antiretrovirals by modulating inflammatory and/or apoptotic cellular mechanisms.[6,7] Mitochondria are the center of energy supply in nearly all body cells by coupling ATP synthesis to oxygen consumption through the oxidative phosphorylation (OXPHOS) system. Specifically, hyperlactatemia can be the result of hypoxic atmospheres or mitochondrial dysfunction which drives energy production out of the mitochondria through anaerobic metabolism and lactic acid generation, which acidifies blood through conversion into lactate and consequent proton release. Blood lactate concentration is the result of lactate production through anaerobic glycolysis (in all body cells but especially on skeletal muscle, liver, nervous and lymphoid system) and its plasmatic clearance by gluconeogenic pathways (mainly on the liver and secondary on the kidney). NRTIs inhibit the unique enzyme responsible for mitochondrial DNA (mtDNA) replication (DNA polymerase γ),[8–10] increase the number of mtDNA mutations and reduce the number of entire mitochondrial genomes. Because mtDNA encodes for 13 proteins of the OXPHOS system responsible for aerobic energy production, important mtDNA depletions can lead to mitochondrial dysfunction moving energy production towards anaerobic metabolism and lactate production. But the complexity of HAART-induced mitochondrial toxicity is however increasing with the description of alternative mechanisms for mitochondrial lesion by NRTIs in absence of mtDNA depletion[11–15] and homeostatic mechanisms able to compensate severe mtDNA depletion and preserve mitochondrial function.[16]

The hypothetic connection between antiretroviral-mediated mitochondrial toxicity and hyperlactatemia was first reported on liver[17–19] and skeletal muscle[17,19–23] as mtDNA depletion and/or OXPHOS system dysfunction. These investigations included however a small number of patients, used invasive approaches and, in most of them, the exploration of mitochondria was partial and limited to mtDNA quantification. Additionally, a correlation between mitochondrial parameters and blood lactate levels was lacking. We herein present the replicational, transcriptional, translational, and biochemical mitochondrial analysis of 26 HIV-infected patients under HAART who developed hyperlactatemia with different degree of clinical severity and lactate levels, both during the hyperlactatemic episode and after clinical recovery, to better assess mitochondrial basis of HAART-related hyperlactatemia. We used a noninvasive method since we studied peripheral blood mononuclear cells (PBMCs). These results have been compared with the values found in nonhyperlactatemic HIV-infected patients on HAART (treated), infected but untreated HIV individuals (naive) and noninfected volunteers (healthy controls).

PATIENTS AND METHODS

Patients

We studied genetic and biochemical PBMC mitochondrial parameters of 26 consecutive HIV-infected patients on HAART undergoing an hyperlactatemic episode (lactate levels above 2 mmol/L) and after clinical recovery. Patients were recruited during 3 years on the Infectious Diseases department of 4 different Catalan hospitals (Hospital Clinic of Barcelona, Hospital Germans Trias i Pujol of Badalona and Hospital Joan XXIII and Hospital of Sant Pau i Santa Tecla from Tarragona) because of increased lactate levels, sometimes accompanied by clinical symptomatology, or because they presented severe accompanying symptomatology with moderate to high lactate values. Patients were categorized in 3 different clinical forms, according to clinical presentation: 13 were asymptomatic, 8 were symptomatic, and 5 had lactic acidosis. We considered as symptoms of hyperlactatemia: fatigue, weakness, abdominal pain, weight loss, tachycardia, and/or dyspnea, after other causes of disease were conveniently discarded. Lactate levels, immunovirologic parameters and one sample of peripheral blood for mitochondrial studies were obtained on admission and after the clinical recovery of hyperlactatemia. Clinical and antiretroviral histories were obtained from the patients' medical records to be correlated with mitochondrial toxicity results. An extensive work-up was performed to exclude other causes of hyperlactatemia. In most of cases hyperlactatemic episode prompted antiretroviral treatment withdrawal that was exclusively restarted, most of times after changing its composition, after clinical recovery and lactate normalization or because of severe immunovirologic reasons.

We compared the results of these hyperlactatemic patients with respect to 3 control groups of subjects that were consecutively collected during the same period of time in the same participant hospitals: 28 nonhyperlactatemic HIV subjects on HAART (treated), 31 HIV-infected but untreated individuals (naive), and 20 uninfected controls (healthy).

Clinical and epidemiological characteristics of included patients and controls are summarized on Table 1. Control group inclusion was made trying to match individual characteristics with those of the hyperlactatemic group of subjects. Treated nonhyperlactatemic patients were matched by sex, age, and time on HIV infection, and time on HAART and time on d-drug treatment (ddI and/or d4T administration) with the hyperlactatemic patients. Naive subjects were matched by sex with hyperlactatemic individuals, but presented statistical significant differences ($P < 0.05$) with respect to age and time on HIV infection. Healthy volunteers presented differences in terms of age and sex with respect the rest of studied groups. All those differences found among our study groups represent, in most of cases, those found in the general population.

All individuals were informed and signed written consent to be included in this protocol that was approved by the Ethical Committee of the Hospital Clinic of Barcelona.

To avoid confounders of mitochondrial toxicity, those patients taking other potentially toxic drugs for mitochondria (ie, aminoglycosides, linezolid, or statins) were excluded from the study, and those subjects with familiar history of mitochondrial disease.

Samples

Mononuclear cells (lymphocytes and monocytes) were isolated by Ficoll density gradient centrifugation[24] and we confirmed a platelet count below 25 per PBMC in all patients coming from the different groups suggestive of negligible platelet contamination.

Protein content was measured according to the Bradford protein–dye binding-based method.[25] Samples were frozen at −80°C until mitochondrial analysis.

Mitochondrial Studies

mtDNA Quantification

Total DNA was obtained by the standard phenol–chloroform extraction procedure. A fragment of the mitochondrial-encoded *ND2* gene and the nuclear-encoded *18S rRNA* gene were amplified in duplicate and separately by quantitative real-time PCR using Lightcycler Roche thermocycler (Roche Diagnostics, Mannheim, Germany), as previously reported.[26,27] The relative content of mtDNA was expressed as the ratio between mtDNA and nDNA amount (*ND2* mtDNA/*18S rRNA* nDNA content).

Mitochondrial RNA Quantification

Total RNA was obtained by an affinity column-based procedure (Rneasy; Qiagen Sciences, Germantown, MD). RNA was reverse-transcribed to cDNA using random hexamer primers and the real-time PCR reaction used to quantify relative mitochondrial cDNA content was performed using Applied Biosystems technology in an ABI PRISM 7700 sequence detection system (Applied Biosystems Inc., Foster City, CA). Quantification of the mitochondrial encoded cytochrome *c* oxidase subunit-II (COX-II) mRNA and the nuclear-encoded housekeeping 18S rRNA were performed using the amplification conditions and the primers previously reported.[27] The relative content of mitochondrial RNA (mtRNA) was expressed as the ratio between mtRNA and nuclear RNA (nRNA) amount (COX-II mtRNA/18S rRNA nRNA content).

Mitochondrial Protein Synthesis

We assessed mitochondrial protein synthesis of the COX-II subunit (mitochondrially encoded, transcribed, and translated) by western blot immunoanalysis.[16,27] This expression was normalized by the content on the mitochondrially located COX-IV subunit (nuclear-encoded and cytoplasmically transcribed and translated) to establish the relative mitochondrial protein expression amount (mtCOX-II/nCOX-IV protein abundance).

Mitochondrial OXPHOS Complexes II, III, and IV (COX) Enzyme Activity

All mitochondrial enzymatic activities were measured spectrophotometrically according to the Rustin et al[28] methodology, slightly modified for complex IV measurement in minute amounts of biological samples.[29] OXPHOS complex II is completely encoded, transcribed and translated by cytoplasmic machinery, whereas CIII and CIV (COX) complexes are partially encoded, transcribed and translated by mitochondrial means. Specific enzymatic activities were expressed in absolute values as nanomols of synthesized substrate or

TABLE 1. Clinical and Epidemiological Characteristics of Included Patients and Controls

	Hyperlactatemic	Treated	Naive	Healthy
Number of subjects (n)	26	28	31	20
Sex (% men)	84.61	83.33	80.64	40 (*)
Age (yrs ± SEM)	46.50 ± 1.86	49.17 ± 2.42	36.33 ± 1.37 (*)	62.80 ± 4.32 (*)
Time infected (mo ± SEM)	118.89 ± 12.90	101.14 ± 14.13	55.26 ± 16.14 (*)	—
Time on HAART (mo ± SEM)	77.54 ± 7.37	62.17 ± 9.66	—	—
At inclusion				
Patients on ddI (%)	25	47	—	—
Patients on d4T (%)	20.8	45	—	—
Patients on ddI + d4T (%)	37.5	6	—	—
Patients without ddI or d4T (%)	16.7	2	—	—

Results are expressed in percentages or as mean value ± SEM. Hyperlactatemic: HIV-infected patients on HAART developing a hyperlactatemic episode; treated: nonhyperlactatemic HIV-infected subjects on HAART; naive: HIV-infected but untreated individuals; healthy: uninfected controls.
*Significant differences ($P < 0.05$) with respect hyperlactatemic-patient's values.

consumed product per minute and milligram of measured protein (nmol/min/mg protein).

Statistical Analysis

The main outcome was the assessment of genetic or biochemical PBMC's mitochondrial parameter change of HIV-infected patients on HAART undergoing a hyperlactatemic crisis (lactate levels over 2 mmol/L) and after clinical recovery and lactate normalization. As mitochondrial genetics parameters we considered mtDNA and mtRNA content and mitochondrial protein synthesis amount. As mitochondrial biochemical parameters we considered those enzymatic activities which take part of the mitochondrial respiratory chain (complexes II, III, and IV).

Additionally, mitochondrial results of hyperlactatemic patients during and after the episode were compared with respect to 3 control groups: nonhyperlactatemic HIV subjects on HAART (treated), HIV-infected untreated individuals (naive), and uninfected volunteers (healthy).

Results were expressed as mean ± standard error of the mean (SEM) or as percentage with respect to healthy controls, the latter were arbitrarily assigned as 100%. We ascertained the normal distribution of mitochondrial and clinical parameters using the Kolmogorov–Smirnov analysis. Parametric *T*-test for independent or paired normal-distributed measures (as needed) were used to search for differences and regression analysis was used to find relationship between quantitative parameters. Otherwise, for nonnormal-distributed parameters, the nonparametric test Mann–Whitney was used to search for independent sample differences, Wilcoxon paired rank test for paired comparisons and Spearman's rank coefficient to search for parameter correlation.

A P value of less than 0.05 was considered significant.

RESULTS

Lactate Levels

Patients suffering from hyperlactatemia on admission had a mean blood lactate value of 3.7 ± 0.6 mmol/L (normal range: 0.8–2 mmol/L). Awareness of early clinical suspicious of hyperlactatemia and routine lactate measurement screening on current HIV clinical management made the lactate level of our patients to be lower with respect to that found in the past or in previous reports,[1–3] and most of our patients were, because of that, asymptomatic (13 of the 26 included). All hyperlactatemic patients were reassessed 9.7 ± 1.2 months later, when blood lactate levels were normalized (1.9 ± 0.1, $P = 0.01$ compared with the baseline values) and they achieved clinical recovery.

Mitochondrial Analyses During Hyperlactatemia and After Clinic Recovery

Hyperlactatemic patients presented all mitochondrial parameters decreased during the hyperlactatemic episode and with respect clinical recovery and lactate normalization (Figs. 1–4). The mitochondrial parameters which showed a most significant improvement after the hyperlactatemic episode resolution were the mitochondrial protein synthesis ($P < 0.05$) and the OXPHOS enzymatic activities of respiratory complexes III and IV ($P < 0.01$ and $P < 0.001$, respectively, Figs. 1, 3, and 4).

Mitochondrial Analyses of Hyperlactatemic Patients Compared With Control Groups

During hyperlactatemia, mtDNA was decreased with respect to the 3 control groups; the comparisons achieved statistical significance with respect to HIV-naive patients ($P < 0.05$) and healthy persons ($P < 0.01$; Fig. 2). This decrease was accompanied by a diminished amount on mitochondrial transcription (mtRNA) and translation species amount (mitochondrial proteins; as shown in Figs. 1 and 2), although the statistical significance was only achieved when hyperlactatemic values were compared with healthy people ($P = 0.01$ and $P < 0.05$, respectively).

The unique parameter which remained nearly equal in all groups was OXPHOS complex II enzymatic activity (Fig. 3). Complex II is not mtDNA encoded rather is entirely encoded by nuclear DNA and entirely transcribed and translated on cytoplasmic ribosomes, and consequently, is supposed to be conserved. Conversely, OXPHOS complexes III and IV function was significantly decreased compared with HIV naive ($P < 0.05$ and $P < 0.01$, respectively) and healthy controls ($P < 0.01$ and $P < 0.05$, respectively; Fig. 3).

If we consider the values found on healthy people as 100%, patients developing hyperlactatemia had a remaining content of 52% of mtDNA, 46% of mtRNA amount, 63% of

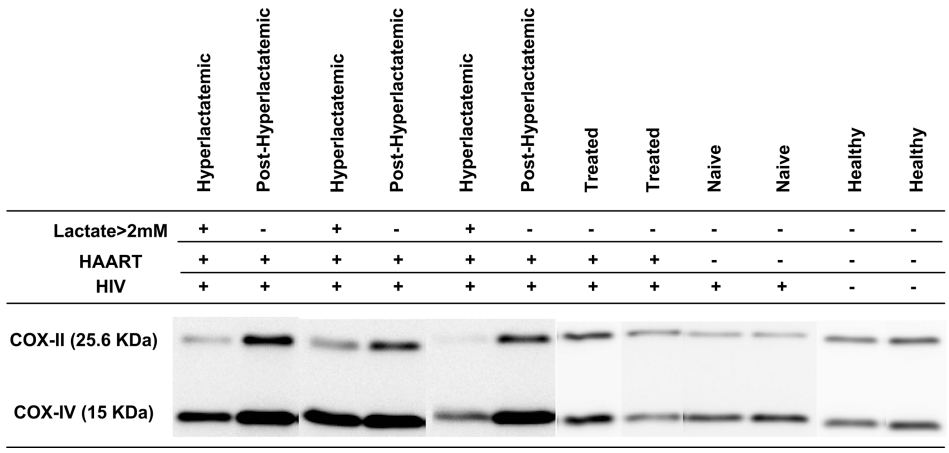

FIGURE 1. Mitochondrial protein expression measured by western blot immunoanalysis of relative mitochondrial-encoded COX-II to nuclear-encoded COX-IV amount on peripheral blood mononuclear cells of studied patients and controls. Hyperlactatemic or posthyperlactatemic: HIV-infected patients on HAART developing a hyperlactatemic episode or after recovery; treated: nonhyperlactatemic HIV-infected subjects on HAART; naive: HIV infected but untreated individuals; healthy: uninfected controls.

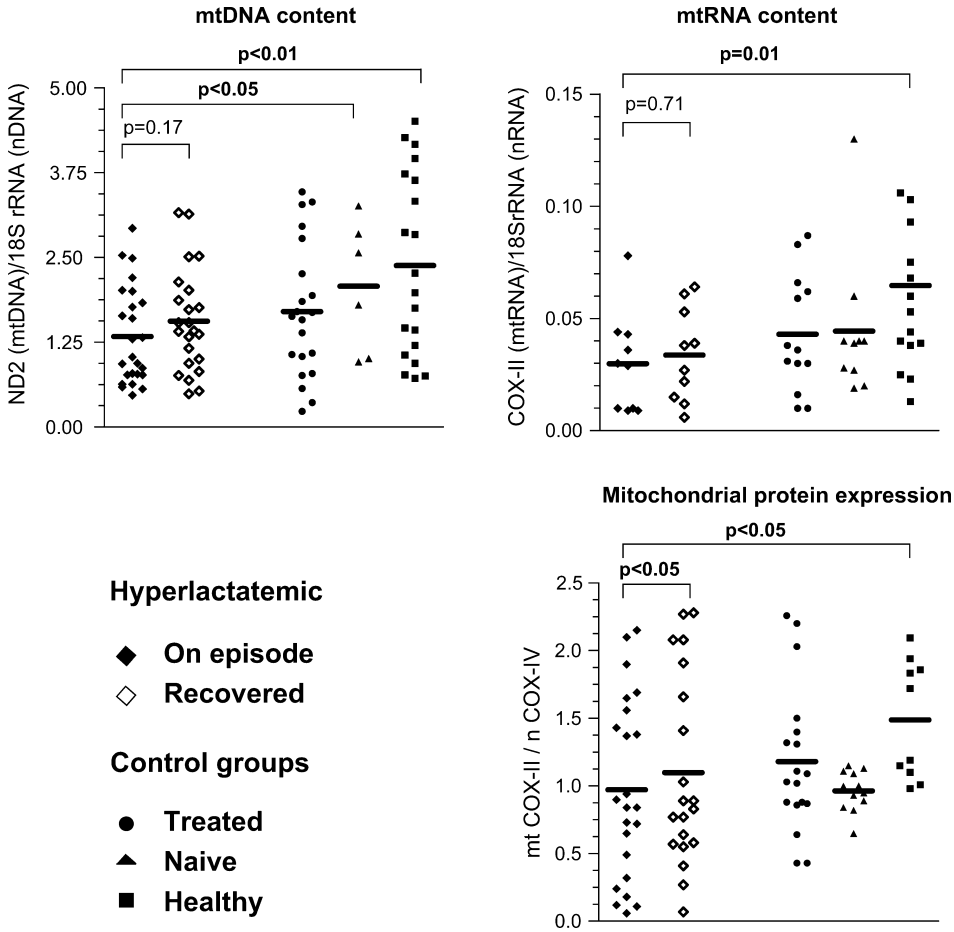

FIGURE 2. Mitochondrial genetic parameters during and after the hyperlactatemic episode with respect 3 different control groups: HAART-treated HIV-infected patients with normal lactate (treated), HIV-infected and untreated patients (naive) and HIV-uninfected volunteers (healthy). Statistical differences between hyperlactatemics during and after the episode have been added (in bold when they are significant), and only those significant differences between hyperlactatemics and the rest of groups.

mitochondrial protein expression quantity and 49% and 69%, respectively, of OXPHOS complex III and IV enzymatic activities (Fig. 4).

Correlation Between Mitochondrial and Clinic Parameters

The comparison for all these mitochondrial parameters according to the subtype of hyperlactatemia (asymptomatic, symptomatic, or lactic acidosis) did not render statistical differences (data not shown), maybe because of the reduced statistical power of so small groups. But when we assessed the relationship between mitochondrial disturbances and the severity of hyperlactatemia measured as blood lactate levels we found that such a relationship existed only for the enzymatic activity of OXPHOS complexes III and IV, which were negatively correlated with lactate concentration ($P < 0.05$ in both cases; Fig. 5), whereas mitochondrial genetic parameters did not ($P = 0.99$ for mtDNA, $P = 0.41$ for mtRNA and $P = 0.38$ for mitochondrial protein expression; data not shown).

DISCUSSION

We found that HAART-related hyperlactatemia is associated with a decrease in all mitochondrial parameters assessed with respect to control values of healthy people and, in some cases (mtDNA and OXPHOS complexes III and IV), also with respect to naive patients. Nonetheless, although mitochondrial parameters were lower than in HIV-infected patients on HAART with normal lactate, none of these differences achieved statistical significance. Interestingly, although all mitochondrial parameters trend to increase after recovery of the hyperlactatemic episode, only mitochondrial translation and OXPHOS complexes III and IV enzymatic activities significantly increase. In addition, although PBMC have been demonstrated a reliable and noninvasive model to perform mitochondrial studies in hyperlactatemic patients, it is foreseeable we can not discard that mitochondrial deficits are bigger in more energy-dependent tissues or those target centers of lactate homeostasis (liver and muscle). Overall, we believe that our findings support the mitochondrial basis for HIV and HAART-related hyperlactatemia.

Mitochondrial toxicity of antiretroviral drugs has been associated mainly with NRTIs use due to its capacity to inhibit mtDNA replication.[4,5] Among dideoxynucleoside analogues, d4T seems to be the most powerful inducer of hyperlactatemia,[19,22,23,30,31] albeit toxic effects of other d-drugs has not been discarded. Most of our hyperlactatemic patients were taking d4T, but most remarkable is the great amount of these hyperlactatemic subjects that were receiving d4T in combination with ddI in comparison to those treated patients who did

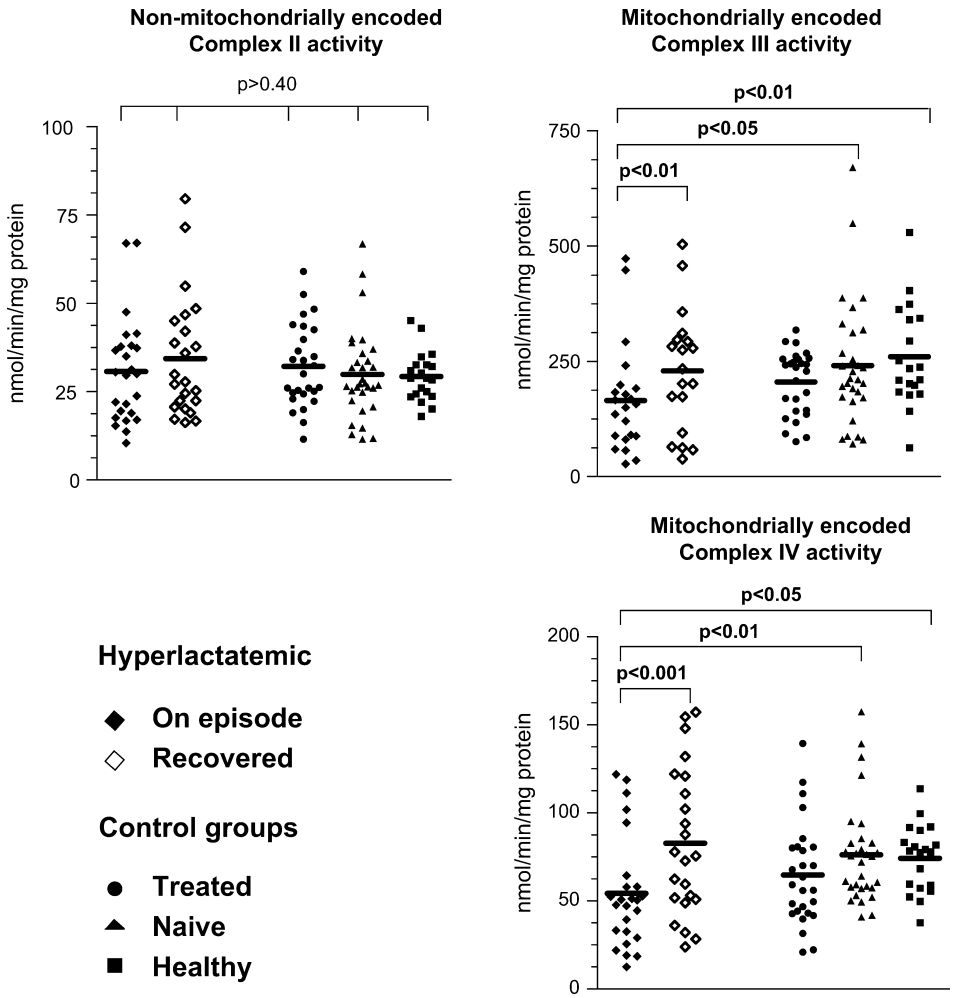

FIGURE 3. Mitochondrial biochemical parameters during and after the hyperlactatemic episode with respect 3 different control groups: HAART-treated HIV-infected patients with normal lactate (treated), HIV-infected and untreated patients (naive), and HIV-uninfected volunteers (healthy). Statistical differences between hyperlactatemics during and after the episode have been added (in bold when they are significant), and only those significant differences between hyperlactatemics and the rest of groups.

not developed the hyperlactatemic disorder. Current guidelines strongly discourage concomitant administration of d4T and ddI, but most of the studied hyperlactatemic patients were included in 2004, when such antiretroviral combination was quite common.

As mtDNA encodes for mitochondrial OXPHOS components, NRTIs-induced mtDNA depletion would led to mitochondrial function impairment. This hypothesis is supported by our data; the finding of mtDNA depletion during hyperlactatemia is associated with a downstream decay of mitochondrial transcription, translation and function. A striking feature of our study is that during the hyperlactatemic episode, mitochondrial biochemistry abnormalities better correlated with blood lactate levels than mitochondrial

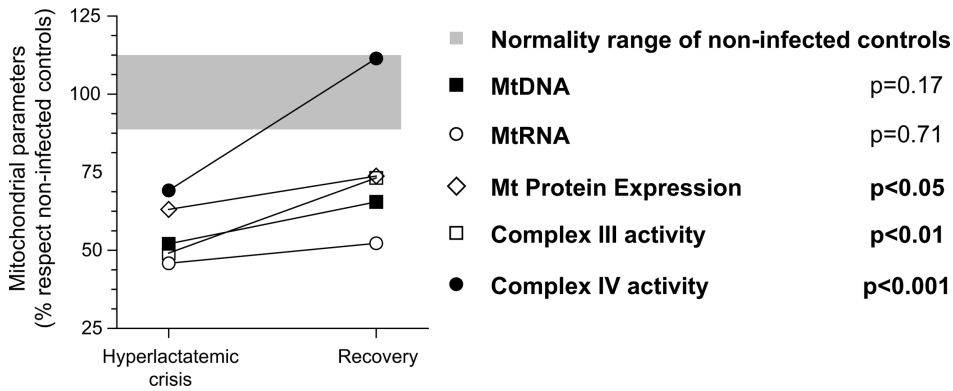

FIGURE 4. Mitochondrial parameters during and after the hyperlactatemic episode expressed as percentages with respect noninfected controls (healthy, arbitrarily assigned 100%). mt protein expression, mitochondrial protein expression.

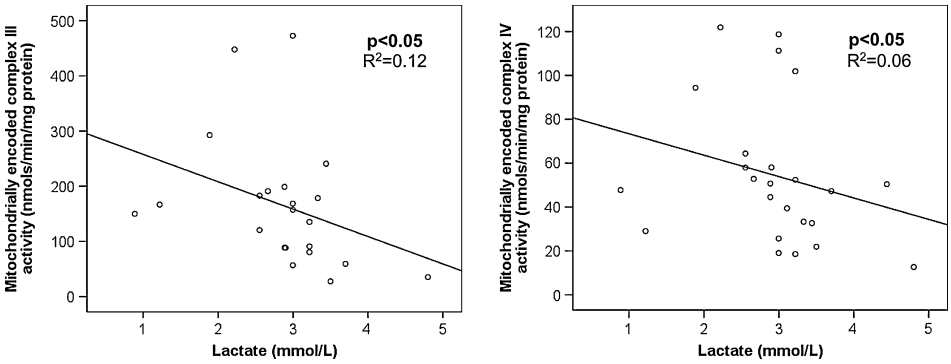

FIGURE 5. Relationship between mitochondrial-encoded biochemical parameters and lactate levels during the hyperlactatemic crisis.

genetics. In agreement with this finding, when blood lactate normalized and clinical recovery was achieved, mitochondrial-encoded OXPHOS complex III and IV enzymatic activities, together with the mitochondrial protein expression, significantly recovered, whereas mitochondrial replication and transcription species amount did not.

The mitochondrial hypothesis of HAART toxicity launched by Brinkman and colleagues in 1998[8] has gained complexity during the last recent years. First, reports show that, even in the absence of mtDNA depletion, NRTIs are able to cause mitochondrial lesion independent to DNA polymerase γ inhibition.[11–15] Second, severe mtDNA depletion induced by NRTIs has been reported to be compensated by mitochondrial transcriptional or translational upregulatory homeostatic mechanisms.[16] These mechanisms could maintain mitochondrial function on adverse circumstances. However, during hyperlactemia, transcription and translation intermediates were decreased, suggesting lack of upregulatory response. Third, assessment of the mitochondrial function has become essential because it is the expected consequence of the genetic lesion and is, ultimately, the responsible of clinical symptoms. Using this overall approach, it has been shown that the change from a highly mitochondriotoxic HAART to other drug schedules with lower toxic potential for mitochondria is first accompanied with a recovery of mitochondrial functions, even if not net changes in mtDNA content are observed.[32,33] This finding suggests that mitochondrial functional recovery antedates the improvement of the genetic lesion or, possibly, that the improvement achieves only one part of the mechanisms disrupted. Both are possible explanations for recovery of mitochondrial function after hyperlactatemia in the absence of substantial mtDNA improvement. But finally we can not discard that mtDNA content of hyperlactatemic patients before the crisis could be so close to the threshold limit value which supports mitochondrial function that the small decay occurred during the hyperlactatemic episode, even not statistically significant, could cross this critical value leading to impaired mitochondrial function. Whatever the explanation is, mitochondrial dysfunction is the final determinant to drive energy production out of the mitochondria towards the cytoplasmatic anaerobic glycolytic pathway responsible of lactate production. All studied patients presented such an increase in blood lactate levels and decreased mitochondrial parameters, but each one of them presented one or more of these parameters especially altered. Consequently with other toxic or genetic mitochondrial diseases that correlate with increased lactate levels but have different mitochondrial parameter impairment etiology, mitochondrial dysfunction of HIV and antiretroviral-induced hyperlactatemia could stand at different genetic, biochemical or synthetic mitochondrial levels, and increased lactate production would just be the common consequence of final mitochondrial impairment.

Although HAART-related hyperlactatemia can be developed in uninfected patients exclusively exposed to antiretroviral therapy,[34] in chronically treated patients' scenario we can not forget the HIV and mitochondria interactions. HIV is able to cause mitochondrial diffuse genetic[5,7] and functional[7,35] lesion by itself that could be mediated by indirect inflammatory or apoptotic mechanisms. The mitochondrial damage present in HIV-infected patients on HAART that underwent an hyperlactatemic crisis could be due to the summatory effect of both HIV-induced damage (also present in naive patients) and mitochondrial toxicity of antiretroviral drugs (also found in nonhyperlactatemic asymptomatic subjects). All these additive adverse effects on mitochondrial function could not be exclusively related to interference of mtDNA replication.

At the present time we can not completely eradicate HIV infection, but we can minimize HIV secondary effects, like mitochondrial lesion, by reducing viral load through antiretroviral administration.[36] Current guidelines advice the beginning of antiretroviral therapy before it did in the past and one of the benefits of early HIV suppression could be avoiding HIV-induced mitochondrial damage. But we have to take care choosing which drugs to use, at which doses and which antiretroviral combinations can be administered together, because the management of all these parameters will also determinate accumulative and chronic mitochondrial damage and future development of adverse clinical events with mitochondrial basis, like hyperlactatemia. Clinicians must be aware of any early sign or symptom of coming toxicities and therapy change could be welcome not only after an hyperlactatemic crisis, but also previously to its development. Although we demonstrate that mitochondrial recovery is possible after an hyperlactatemic episode, it is essential to prevent secondary effects of HAART better than managing them. Once hyperlactatemia is developed, early management

of all disturbances and normalization of lactate and acidemia, will help in mitochondrial and clinical recovery achievement.

Currently available information about HAART-related adverse event etiology has moved antiretroviral guidelines to less potent mitotoxic drug administration, which has fortunately reduced associated mitochondrial damage and derived adverse events, like hyperlactatemia. In developed countries these strategies consist on reducing antiretroviral doses, changing HAART-schedules to nucleoside-sparing regimens or guiding patients to structured-treatment interruptions,[33,37–40] but scarce work has been done to evaluate strategies which actively reverts mitochondrial induced damage, even in the context of concomitant antiretroviral administration, as mitochondrial drug therapy.[41] Further studies should be addressed to assess how to prevent or correct mitochondrial function in HIV-infected and HAART-treated symptomatic or asymptomatic patients but also to find premature toxicity markers that would allow us avoiding adverse effects of chronic HIV infection and treatment. The performance of mitochondrial assays in noninvasive and easy-obtaining samples (as mononuclear cells) based on measuring functional parameters and non-exclusively limited on measuring mtDNA content could be a useful tool for these screenings.

ACKNOWLEDGMENTS

We are in debt with many people who has contributed to the present work by providing us their valuable help. Among them: Sònia López, Mireia Nicolàs, Jordi Guallart, Maria Larrousse, Ágat León, Ángel Ballesteros, Joaquim Peraire, Sergi Veloso, Consuelo Viladés, and Elisabet Deig.

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Histological and molecular features of lipomatous and nonlipomatous adipose tissue in familial partial lipodystrophy caused by LMNA mutations

Revista: Clinical Endocrinology (Oxf). 2012 Jun; 76(6):816-24. doi: 10.1111/j.1365-2265.2011.04208.x

PMID: 21883346

Título: Características histológicas y moleculares del tejido adiposo lipomatoso y no lipomatoso en la lipodistrofia parcial familiar de tipo 2 causada por mutaciones en LMNA

La lipodistrofia familiar de tipo 2 (FPLD2) es una mutación en el gen LMNA que provoca una rara enfermedad en el tejido adiposo, en la cual en ocasiones aparecen lipomas ocasionalmente. En este estudio, se pretendió caracterizar histológicamente los lipomas asociados a FPLD2 y estudiar la expresión de genes y proteínas relacionadas con el control del ciclo celular, función mitocondrial, inflamación y adipogénesis. Los adipocitos observados de los pacientes lipodistróficos eran significativamente mayores que los controles tanto en lipomas como en la zona periférica de los lipomas. El tejido adiposo lipodistrófico presentaba infiltración de macrófagos CD68+ y linfocitos CD3+. La expresión de TP53 estaba reducida en todos los tipos de lipomas. A nivel proteico C/EBPβ, p53 y pRβ aparecían severamente alterados en lipomas y perilipomas, mientras la expresión de C/EBPα era normal. Los genes de función mitocondrial se expresaban menos en la grasa lipoatrófica. En los lipomas y los perilipomas la expresión de genes adipogénicos era inferior que en controles. Se concluyó que incluso en los lipomas la maquinaria adipogénica está alterada. En las zonas lipoatróficas de pacientes FPLD2 el fenotipo histológico es casi normal, exhibiendo una inflamación de bajo grado. Estos resultados sugieren que la vía p53 y algunas proteínas adipogénicas como C/EBPα pueden contribuir a mantener este estado casi normal del tejido adiposo de los pacientes.

Clinical Endocrinology (2012) **76**, 816–824 doi: 10.1111/j.1365-2265.2011.04208.x

ORIGINAL ARTICLE

Histological and molecular features of lipomatous and nonlipomatous adipose tissue in familial partial lipodystrophy caused by LMNA mutations

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Summary

Objectives Type 2 familial partial lipodystrophy (FPLD2) is a rare adipose tissue (AT) disease caused by mutations in LMNA, in which lipomas appear occasionally. In this study, we aimed to histologically characterize FPLD2-associated lipomatosis and study the expression of genes and proteins involved in cell cycle control, mitochondrial function, inflammation and adipogenesis.

Design and patients One lipoma and perilipoma fat from each of four subjects with FPLD2 and 10 control subjects were analysed by optical microscopy. The presence of inflammatory cells was evaluated by immunohistochemistry. Real-time RT-PCR and Western blot were used to evaluate gene and protein levels.

Results Adipocytes from lipodystrophic patients were significantly larger than those of controls, in both the lipomas and perilipoma fat. Lipodystrophic AT exhibited $CD68^+$ macrophages and $CD3^+$ lymphocytes infiltration. TP53 expression was reduced in all types of lipomas. At protein level, C/EBPβ, p53 and pRb were severely disturbed in both lipodystrophic lipomas and perilipoma fat coming from lipoatrophic areas, whereas the expression of CEBPα was normal. Mitochondrial function genes were less expressed in lipoatrophic fat. In both lipomas and perilipoma fat from lipoatrophic areas, the expression of adipogenes was lower than controls.

Conclusions Even in lipomas, the adipogenic machinery is impaired in lipodystrophic fat coming from lipoatrophic regions in FPLD2, although the histological phenotype is near-normal, exhibiting low-grade inflammatory features. Our results suggest that the p53 pathway and some adipogenic proteins, such as CEBPα, could contribute to the maintenance of this near normal phenotype in the remnant AT present in these patients.

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(Received 13 May 2011; returned for revision 30 May 2011; finally revised 16 August 2011; accepted 17 August 2011)

Introduction

Type 2 familial partial lipodystrophy (FPLD2; OMIM 151660) is characterized by a loss of subcutaneous adipose tissue (AT) in the limbs and buttocks, accumulation of fat in the neck and face and predisposition to insulin resistance, leading to complications such as glucose intolerance, dyslipidaemia, liver steatosis and increased risk of coronary heart disease.[1] FPLD2 results from mutations in the LMNA gene,[2] which codes for several spliced proteins, including lamin A, lamin C, lamin C2 and lamin AΔ10. Lamin A, a key component of the nuclear lamina, is formed from post-translational modifications of a precursor protein, prelamin A.[3]

Although it has not been frequently reported in the literature,[4] in our clinical experience, lipomas can arise in patients with FPLD2 (20% of prevalence in our cohort), even in lipoatrophic areas. Lipomas are common benign neoplasms composed of mature fat cells with minimal or no variation in shape and size and no nuclear atypia.[5] The aetiology of lipomas remains obscure, though some germ-line mutations in the RB1 gene [6] and mitochondrial DNA ($tRNA^{Lys}$) [7,8] among other genes [9] have been associated with multiple lipomatosis, and chromosomal aberrations have been described in lipoma adipocytes.[5] However, details of the cellular and molecular phenomena underlying the pathogenesis of this kind of tumours remain unclear.

Among the few studies published on the molecular basis of lipomas, altered expression of the master transcription factors of adipogenesis (PPARγ and C/EBPα) has been reported.[10,11] On the other hand, some features of brown fat have been reported in lipomas associated with $tRNA^{Lys}$ mutations.[10] This last aspect is intriguing because fat accumulates in the neck, axillas and back of patients with FPLD2 just the usual location of brown AT in the human beings.[12]

To the best of our knowledge, the histological and molecular features of FPLD2-associated lipomas have not yet been studied.

Subjects and methods

This study was approved by the Ethics Review Panel of the Consellería de Sanidade (Xunta de Galicia, Spain) and carried out according to the ethical guidelines of the Declaration of Helsinki. All of the patients provided informed consent for participation in the study and publication of their clinical, biochemical and genetic information.

Subjects

Four patients with FPLD2 and lipomas were studied. Two of the patients had a known mutation in the LMNA gene (R482W), and the other two had a not yet described mutation in the same gene (I299V). Ten age- sex- and BMI-matched nonlipodystrophic subjects with lipomas were studied as controls. The anthropometric measures, clinical features, anatomic location and size of the lipomas in these subjects are shown in Table 1.

LMNA mutation analysis

DNA was prepared from peripheral white blood cells using standard procedures.[13] LMNA exons 1–12 and the surrounding intronic sequences were amplified by PCR using primers and conditions that were described previously.[14] The fragments were sequenced using the ABI PRISM BigDye Terminator v3.0 Ready Reaction Cycle Sequencing kit (PE Applied Biosystems, Carlsbad, CA, USA).

Table 1. Clinical characteristics of the subjects

				Lipoma					
Subject	Gender	Age	BMI	FPLD2	Location	Size (cm)	DM2	HBP	CVD
1	M	66	25·5	R482W	Back	7·0 × 6·4	No	No	Stroke
2	F	40	24·9	R482W	Abdomen	4·3 × 3·0	No	No	No
3	F	43	32·3	I299V	Thigh	3·0 × 2·1	Yes	Yes	No
4	F	70	33·4	I299V	Abdomen	4·8 × 4·4	Yes	Yes	No
5	F	59	30·2	NO	Back	7·0 × 5·5	No	No	No
6	F	53	27·4	NO	Back	5·0 × 5·0	No	Yes	No
7	F	59	28·9	NO	Thigh	4·0 × 2·5	Yes	No	No
8	M	28	21·5	NO	Abdomen	6·0 × 4·0	No	No	No
9	F	29	23·8	NO	Back	4·0 × 3·0	No	No	No
10	F	25	21·1	NO	Back	6·5 × 5·3	No	No	No
11	F	31	28·8	NO	Thigh	3·5 × 4·0	No	No	No
12	F	53	33·7	NO	Back	6·5 × 6·0	IFG	No	No
13	F	66	30·0	NO	Abdomen	4·0 × 3·5	Yes	Yes	No
14	M	28	29·6	NO	Thigh	4·5 × 4·0	No	No	No

M, male; F, female; BMI, body mass index (kg/m^2); DM2, type 2 diabetes mellitus; IFG, impaired fasting glycaemia; HBP, high blood pressure; CVD, cardiovascular disease; FPLD2, type 2 familial partial lipodystrophy.
All of lipomas were encapsulated.

Lipoma resection and perilipoma fat biopsy

One lipoma and a sample of nonlipomatous AT close to the tumour were extirpated from lipodystrophic and nonlipodystrophic subjects following standard surgical procedures. From one of the FPLD2 patients (R482W, women), two additional fat samples were obtained from the neck and thigh. Fat samples were cleaned of visible fibrous connective tissue and blood vessels and divided into roughly equal portions. One portion was snap-frozen immediately in liquid nitrogen and stored at −80 °C until further analysis, and the other was fixed in 10% buffered formalin to be processed for optical microscopy.

Optical microscopy

The fixed samples were embedded routinely in paraffin. Sections (4 μm thick) were stained with haematoxylin–eosin (HE) and analysed using light microscopy (Olympus BX51, Hamburg, Germany). For the morphometric study, 30 randomly selected adipocytes were analysed per sample. The major and minor diameters of each adipocyte were measured using an ocular micrometre.

Immunohistochemistry

The sections were mounted on FLEX IHC slides (Dako, Glostrup, Denmark) and heated at 60 °C for 1 h. The technique was automatically performed using an Autostainer Link 48 (Dako). After deparaffination and epitope retrieval in EnVision FLEX target retrieval solution with high pH for 20 min at 97 °C, the slides were allowed to cool in PT Link to 65 °C, and then in Dako wash buffer for 5 min at room temperature. The immunostaining protocol included successive incubation in EnVision FLEX peroxidase-blocking reagent (5 min); primary ready-to-use FLEX antibodies against CD68 (PG-M1), CD3, CD20, CD138 and Ki67; EnVision FLEX + mouse (linker) (15 min); EnVision FLEX/HRP (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-mouse immunoglobulins, 20 min); substrate working solution (mix) (3,3′-diaminobenzidine tetrahydrochloride chromogen solution, 10 min); and EnVision FLEX haematoxylin (9 min). The proliferation index measured by Ki67 was estimated using ACIS III Image Analysis (Dako).

Western blot

Whole-cell lysates were obtained from human AT by sonication in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0·5% sodium deoxycholate, 0·1% SDS and 50 mM Tris, pH 8) containing 0·5 mM DTT, 1 mM PMSF, 50 mM NaF, 1 mM Na_3VO_4 and protease inhibitor cocktail (Sigma, St Louis, MO, USA) at 4 °C. Two alternative protein extraction methods were used for the Lamin A/C detection assays: (i) final pellets (insoluble material) obtained with the lysis buffer mentioned above were solubilized using hot (100 °C) loading buffer for 1 min or (ii) AT samples were lysed in 'hot' lysis buffer (90 °C) containing 20 mM Tris–HCl (pH 7·5), 1% SDS, 1 mM Na_3VO_4, 1 mM PMSF, 5% beta-mercaptoethanol and protease inhibitors. Proteins were resolved in 8% or 10% SDS–

PAGE gels and transferred to PVDF membrane (Millipore, Bedford, MA, USA). Blots were blocked for 1 h at room temperature in 2% or 5% nonfat milk in PT (PBS, 1% Tween-20). The blots were probed with anti-PPARγ (sc-7196), anti-PPARγ2 (sc-22020), anti-C/EBPβ (sc-150), anti-p21 (sc-397), anti-p53 (sc-65334) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-C/EBPα (#2843), anti-phospho-Rb (#3590) (Cell Signaling Technology Inc., Danvers, MA, USA), anti-Lamin A/C (monoclonal MANLAC1 4A7, kindly provided by Prof. G.E. Morris) [15] and monoclonal anti-β-actin (A5316) (Sigma Aldrich, St Louis, MO, USA). Anti-rabbit IgG peroxidase conjugated (Thermo Scientific, 32460, Rockford, IL, USA) or anti-mouse IgG peroxidase conjugated (GE Healthcare, Piscataway, NJ, USA, NA931V) were the secondary antibodies. Immunoblot bands were revealed by chemiluminescence (Supersignal West Dura extended duration substrate; Thermo Scientific). Densitometry was performed using ImageJ software.[16]

Real-time RT-PCR

Expression of LMNA, UCP1 (a brown fat marker), DLK1 (Pref1, a preadipocyte marker), CEBPA, CEBPB, SREBF1, PPARG, LPL, SLC2A4 (GLUT4), LEP, FABP4 (all of them are genes involved in adipocyte differentiation), RB1, TP53 (p53) (genes involved in cell cycle control), CD3D, CD68, IL8, TNF (TNFα) (inflammation markers), MT-CO2, COX4I1 (COX4) and SIRT3 mRNAs (genes related with mitochondrial function) and the internal control RNA polymerase II (RPII) were quantified in a thermal cycler (Light Cycler 2.0; Roche Diagnostics, Sant Cugat del Valles, Spain) as described previously.[17] Mitochondrial DNA (mtDNA) was quantified by quantitative real-time PCR as described previously [18] and expressed as relative levels with respect to the nuclear, single-copy CEBPA.

Statistical analysis

Data are provided as means (SD). Owing to the low number of experiments, data were considered as not normally distributed, and the differences between groups were evaluated by a nonparametric test (Mann–Whitney *U*-test). Significance was set at $P < 0{\cdot}05$. All statistical analyses were carried out using SPSS 14.0 software (Chicago, IL, USA).

Results

Of the four lipomas, two were from patients from the same pedigree [19] with FPLD2 because of the R482W mutation in LMNA, whereas the other two came from a mother and daughter from another pedigree (Fig. 1a,b) with a previously not described missense mutation in LMNA (NM_170707.2): c. 895 A>G, p. Ile299Val (Fig. 1c). This mutation was not found in 100 control chromosomes.

Two of the lipomas were from nonlipoatrophic areas [back (R482W) and abdomen (I299V)], and two from lipoatrophic areas [abdomen (R482W) and thigh (I299V)].

Histological findings and immunohistochemistry

All of the studied lipomatous tissue stained with HE exhibited the typical aspects of white AT, with nonhistological features of brown fat. This tissue was similar to the perilipoma AT for both lipodystrophic and control samples (Fig. 2a).

The morphometric study revealed that adipocytes from lipomas were significantly larger than adipocytes from perilipoma fat in both controls and lipodystrophic patients. Unexpectedly, the lipodystrophic adipocytes were significantly larger than control adipocytes (Fig. 2b).

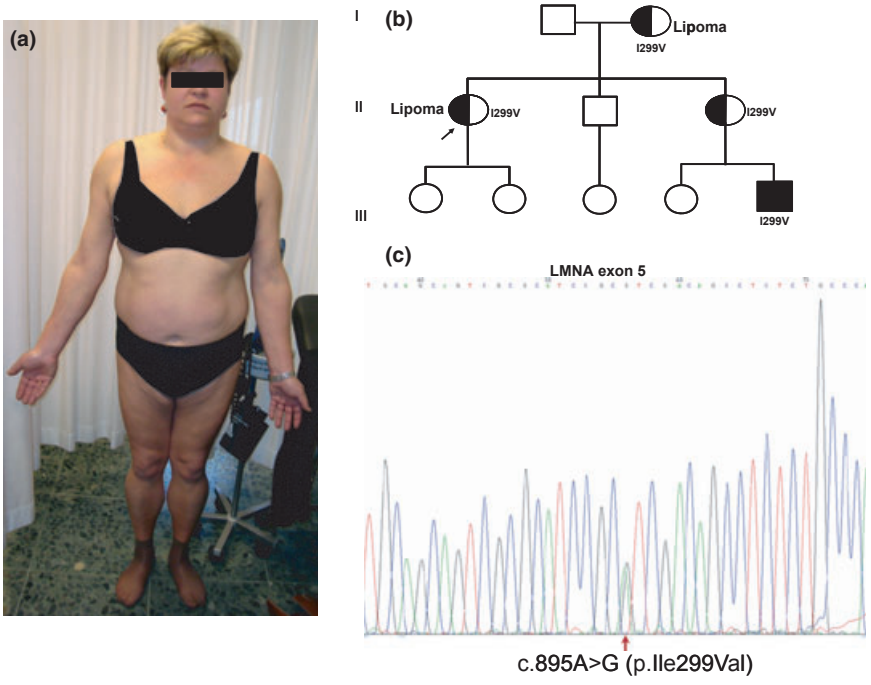

Fig. 1 (a): Photo of the index case with type 2 familial partial lipodystrophy (FPLD2) bearing the I299V mutation in LMNA. This patient showed an atypical form of FPLD with a loss of fat in the buttocks and limbs, abdominal fat accumulation, muscular hypertrophy, severe insulin resistance, diabetes mellitus, hypertriglyceridaemia and hypoleptinaemia. (b) Pedigree of the family with the I299V mutation in LMNA. The arrow indicates the index case. The mother and sister of the index case have a similar fat distribution to that of the propositus. The nephew of the index case is asymptomatic. The mother suffers diabetes mellitus with severe insulin resistance, hypertriglyceridaemia and hypoleptinaemia, whereas the sister has a normal glucose tolerance, hypertriglyceridaemia and normal leptinaemia. (c) Genetic analysis of LMNA in the proband, indicating a heterozygous A to G transition at position 895 (exon 5), predicting an I299V substitution.

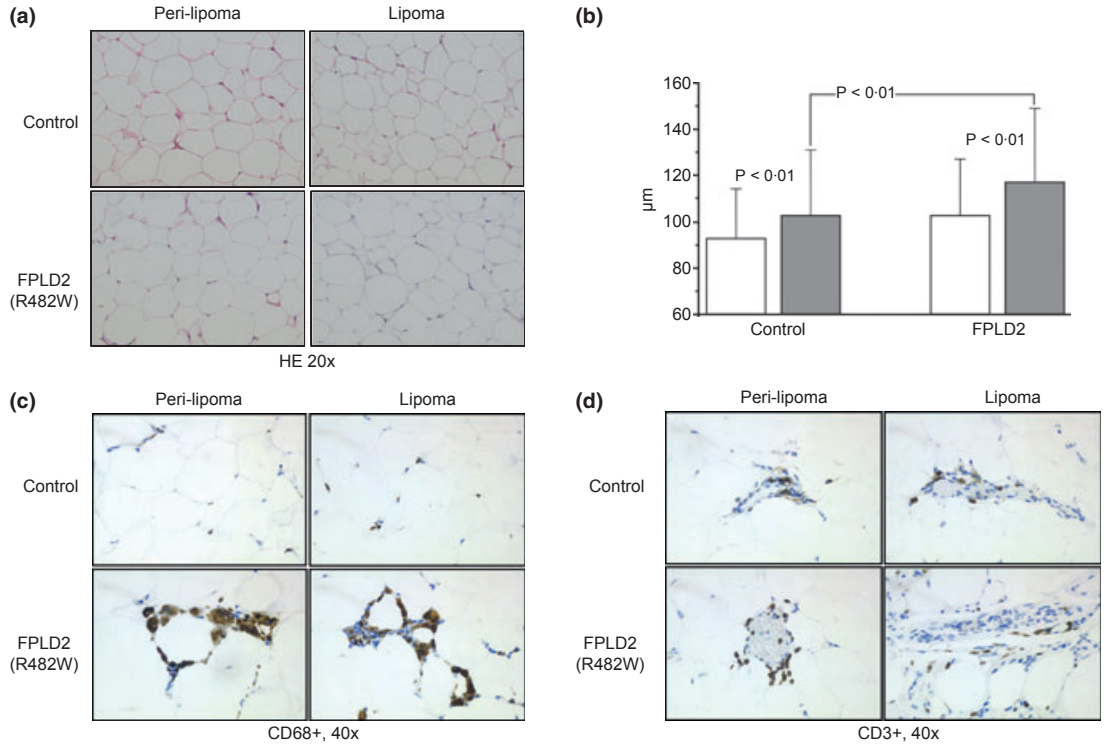

Fig. 2 Histological and immunohistochemical findings in representative perilipoma and lipoma samples. (a) Haematoxylin-eosin staining showing the typical white adipose tissue (AT) appearance in both tissues. (b) Morphometric study. Adipocytes from lipomas (grey bars) were larger than those from perilipomatous fat (white bars), and adipocytes from lipodystrophic samples were larger than those from control fat samples. (c) Immunohistochemistry for CD68. $CD68^+$ cells surrounded several adipocytes in the crown-like structures frequently seen lipodystrophic AT. (d) Immunohistochemistry for CD3. More $CD3^+$ lymphocytes appeared in lipodystrophic lipomas with a scattered and perivascular distribution. In the rest of the samples, the $CD3^+$ T cells were scarce with perivascular distribution.

$CD68^+$ macrophages were found in both lipomas and perilipoma AT from lipodystrophic subjects, but they were very rare in control subjects, and the distribution was preferentially scattered. In lipodystrophic samples, these cells appeared to be both scattered and to surround adipocytes. Crown-like structures, a feature of adipocyte phagocytosis by infiltrating macrophages, were also observed in these patients. This disposition around adipocytes seems to be more frequent in lipomas than perilipoma fat in lipodystrophic patients (Fig. 2c).

Apparently, more $CD3^+$, with a scattered and perivascular distribution, were found in lipodystrophic lipomas compared with the other samples. In the rest of the samples, the $CD3^+$ T cells were scarce with perivascular distribution (Fig. 2d).

The proportion of $Ki67^+$ proliferating cells was significantly higher in lipomas from lipodystrophic patients than those from control subjects ($2{\cdot}75 \pm 0{\cdot}96\%$ *vs* $0{\cdot}25 \pm 0{\cdot}5\%$, $P < 0{\cdot}007$).

B cells ($CD20^+$) and plasma cells ($CD138^+$) were essentially absent in all of the analysed samples.

Real-time RT-PCR

The gene expression results are shown in Table 2. Each individual lipodystrophic fat sample was compared with control samples coming from the same anatomical region.

Among genes that are involved in cell cycle control (RB1 and TP53), no differences were found between perilipoma AT from lipodystrophic subjects and perilipoma AT from control subjects. In lipomas, the most remarkable data were the lower expression of TP53 from control and lipodystrophic subjects compared with perilipoma AT.

We also analysed markers of mitochondrial function, and the expression of MT-CO2 mRNA, a transcript encoded by mitochondrial DNA, was significantly reduced in perilipoma AT from the FPLD2 patients compared to controls, with a less marked tendency to a lower expression in lipodystrophic lipomas. This reduction could not be attributed to changes in the relative amounts of mitochondrial DNA, which were unaltered or even increased in the lipodystrophic samples. SIRT3, a mitochondrial protein involved in the regulation of mitochondrial lipid catabolism,[20] was clearly reduced only in lipomas from FPLD2 patients.

We also analysed the expression of inflammatory markers and infiltrating white blood cells genes. Regarding CD3D and CD68 transcript expression, no clear tendency was found in the analysed samples supporting our immunohistochemical findings. It may happen that the small representation of RNA from the $CD3^+$ and $CD68^+$ cells in the overall preparations of RNA from total AT samples give a low sensitivity to this assays respect to immunohistochemical observations. However, it is worth to mention that the expression of both IL8 and TNF was higher in lipoatrophic perilipoma samples compared with controls. Moreover, a tendency to a lower expression of these genes was found in lipodystrophic lipomas.

Table 2. Gene expression relative to RPII gene in adipose tissue from perilipoma and lipoma biopsies of subjects with FPLD2 and controls

Adipose tissue from lipoatrophic areas

	Perilipoma (Abdomen)		Perilipoma (Thigh)		Lipoma (Abdomen)		Lipoma (Thigh)	
Gene	Control	R482W	Control	I299V	Control	R482W	Control	I299V
RB1	1·04 ± 0·21	1·34	1·01 ± 0·11	1·44	0·74 ± 0·05	1·33	0·87 ± 0·04	0·99
TP53	1·01 ± 0·24	1·20	1·00 ± 0·90	1·23	0·56 ± 0·22	1·03	0·64 ± 0·04	1·01
LMNA	1·02 ± 0·37	1·34	1·04 ± 0·33	1·27	0·66 ± 0·09	1·05	1·02 ± 0·50	1·03
SREBF1	1·05 ± 0·37	0·81	1·15 ± 0·66	0·35	3·52 ± 0·19	1·24	1·94 ± 0·21	0·93
PPARG	1·02 ± 0·21	0·45	1·02 ± 0·18	0·32	0·86 ± 0·33	0·38	1·03 ± 0·13	0·10
CEBPA	1·02 ± 0·22	0·31	1·04 ± 0·30	0·36	1·28 ± 0·52	0·58	1·95 ± 0·69	0·14
CEBPB	1·04 ± 0·31	0·18	1·08 ± 0·45	0·47	0·93 ± 0·04	0·71	1·22 ± 0·70	0·45
LPL	1·03 ± 0·25	0·38	1·07 ± 0·27	0·27	0·50 ± 0·33	0·38	0·98 ± 0·28	0·10
SLC2A4	1·03 ± 0·29	0·45	1·04 ± 0·31	0·23	2·44 ± 1·07	1·00	2·74 ± 0·81	0·26
LEP	1·46 ± 1·23	0·87	1·14 ± 0·47	1·17	0·95 ± 0·80	2·66	2·17 ± 1·31	0·20
FABP4	1·04 ± 0·33	0·53	1·01 ± 0·16	0·43	0·80 ± 0·01	0·78	1·28 ± 0·55	0·13
CD3D	1·08 ± 0·06	0·40	0·76 ± 0·19	1·14	0·97 ± 0·11	0·50	1·06 ± 0·52	1·38
CD68	1·04 ± 0·13	0·68	0·90 ± 0·06	0·74	1·14 ± 0·01	0·63	1·04 ± 0·2	3·93
IL8	1·07 ± 0·87	1·57	0·98 ± 0·06	2·30	1·21 ± 0·91	0·79	2·54 ± 2·04	0·11
TNF	1·36 ± 0·32	1·84	0·70 ± 0·24	3·94	0·91 ± 0·25	0·35	0·76 ± 0·53	0·92
MT-CO2	1·11 ± 0·31	0·61	1·51 ± 0·14	0·63	0·95 ± 0·35	0·88	1·03 ± 0·50	0·38
COX4I1	1·08 ± 0·05	0·77	0·62 ± 0·19	1·06	0·51 ± 0·22	1·02	0·94 ± 0·32	0·73
SIRT3	1·05 ± 0·09	0·81	1·16 ± 0·66	1·09	1·48 ± 0·12	0·71	0·92 ± 0·17	0·32
mtDNA	1·04 ± 0·04	0·31	0·65 ± 0·07	0·98	1·14 ± 0·05	1·43	1·2 ± 0·57	1·20

Adipose tissue from lipohypertrophic areas

	Perilipoma (Back)		Perilipoma (Abdomen)		Lipoma (Back)		Lipoma (Abdomen)	
	Control	R482W	Control	I299V	Control	R482W	Control	I299V
RB1	1·03 ± 0·20	1·03	1·03 ± 0·28	0·98	1·04 ± 0·41	1·02	0·74 ± 0·05	1·16
TP53	1·02 ± 0·19	0·82	1·02 ± 0·24	1·32	0·56 ± 0·60	0·59	0·58 ± 0·22	0·52
LMNA	1·10 ± 0·03	1·10	1·05 ± 0·37	1·81	0·72 ± 0·25	0·49	0·66 ± 0·09	0·57
SREBF1	1·02 ± 0·21	1·22	1·05 ± 0·37	3·09	1·14 ± 0·49	1·23	3·52 ± 0·19	2·29
PPARG	1·07 ± 0·38	0·71	1·02 ± 0·21	0·89	0·81 ± 0·43	0·73	0·86 ± 0·33	0·76
CEBPA	1·09 ± 0·42	0·82	1·02 ± 0·22	1·08	1·46 ± 0·76	1·48	1·28 ± 0·52	1·43
CEBPB	1·35 ± 1·13	0·67	1·04 ± 0·31	0·74	0·88 ± 0·38	0·81	0·93 ± 0·04	0·83
LPL	1·09 ± 0·40	0·66	1·03 ± 0·25	0·83	0·80 ± 0·40	0·84	0·50 ± 0·33	0·42
SLC2A4	1·20 ± 0·51	0·51	1·03 ± 0·29	0·66	2·27 ± 1·29	1·25	2·44 ± 1·07	0·89
LEP	1·09 ± 0·40	0·66	1·03 ± 0·25	0·83	0·80 ± 0·40	0·84	0·50 ± 0·33	0·42
FABP4	1·05 ± 0·37	1·42	1·04 ± 0·33	0·62	1·42 ± 0·39	1·60	0·80 ± 0·01	0·97
CD3D	1·55 ± 0·69	1·67	0·97 ± 0·11	0·67	0·9 ± 0·16	0·91	0·62 ± 0·07	0·45
CD68	1·19 ± 0·47	0·69	1·06 ± 0·11	0·51	0·88 ± 0·28	1·30	1·00 ± 0·40	0·71
IL8	1·01 ± 0·33	1·04	0·50 ± 0·03	0·41	1·46 ± 1·68	0·09	0·49 ± 0·10	0·17
TNF	0·88 ± 0·56	0·12	0·56 ± 0·47	0·69	1·22 ± 1·17	0·44	0·81 ± 0·17	0·62
MT-CO2	0·97 ± 0·26	0·24	0·92 ± 0·11	0·58	1·34 ± 0·91	1·47	0·75 ± 0·12	0·71
COX4I1	0·99 ± 0·93	0·52	0·98 ± 0·04	1·09	0·95 ± 0·29	1·57	0·42 ± 0·02	0·29
SIRT3	1·05 ± 0·24	0·95	0·91 ± 0·01	1·59	1·24 ± 0·56	0·59	1·17 ± 0·31	0·81
mtDNA	0·43 ± 0·05	1·55	0·91 ± 0·12	1·30	1·14 ± 0·6	1·32	1·36 ± 0·86	1·23

RPII, RNA polymerase II; FPLD2, type 2 familial partial lipodystrophy.

The expression of genes directly related to adipocyte differentiation was significantly reduced in perilipoma lipoatrophic AT from patients with FPLD2. Thus, the relative expression of the CEBPA, CEBPB, PPARG, LPL and SLC2A4 genes was 45–80% lower in these patients. However, these differences were not found in the lipodystrophic samples coming from lipohypertrophic areas.

The expression of several adipogenes was increased in lipomas. The expression of SREBF1, CEBPA and SLC2A4 was increased in lipomas from control subjects compared with perilipoma AT. Contrary to expectations, no changes were observed in the remaining adipogenes (PPARG, CEBPB, LPL, LEP, FABP4). In general terms, the expression of the analysed adipogenes in lipodystrophic lipo-

mas coming from lipoatrophic areas was reduced; however, this was not found in lipodystrophic lipomas coming from lipohypertrophic areas.

The expression of UCP1 and Pref1 was negligible in all of the studied samples.

Western blot

The levels of lamin A and lamin C were severely reduced in the lipodystrophic AT (perilipoma and lipoma) from lipoatrophic body areas, but not in the AT from lipohypertrophic areas (back) (Fig. 3a). To corroborate that the anatomical location was the cause of these findings, AT from different parts of the body (neck, abdomen and thigh) of the same FPLD2 patient (R482W) was analysed for lamin A/C. We found a low level of lamin A independent of anatomical location, though mainly in the lipoatrophic areas. We also detected an extra band corresponding to prelamin A accumulation in the same lipodystrophic patient (Fig. 3b). The only difference that we found among these lipodystrophic patients was gender. In the only man we studied, the levels of lamin A in both lipoma and perilipoma fat were very similar as those of control subjects.

Because these results were not concordant with our previous studies,[17] we repeated the analysis using a different protein extraction protocol that included heating the AT samples in the lysis buffer at 90 °C and increasing the reducing conditions. The levels of lamin A/C after this protocol were similar to those found in control subjects (Fig. 3c). To determine the possible causes of the low lamin A levels in lipodystrophic patients, we evaluated the amount of protein in the insoluble pellet, observing a clear lamin A band for the samples from the woman with FPLD2 (R482W), but not in the soluble fraction, indicating a lower solubility (Fig. 3d).

Analysis of the major transcription factors controlling adipogenesis showed a marked reduction of the CEBPβ levels in the lipodystrophic AT, both lipomatous and perilipomatous, mainly in samples from lipoatrophic areas (Fig. 4a). The total amount of PPARγ was also reduced in both lipodystrophic samples and non-lipodystrophic lipomas, and no significant differences were found in the levels of CEBPα (Fig. 4b). As PPARγ2 is preferentially expressed in AT, we also analysed this isoform. Only the sample coming from the thigh of a FPLD2 (I299V) patient showed a lower expression of this protein (Fig. 4b).

Regarding the transcription factors controlling cell cycle, we found that the expression of phosphorylated pRb was markedly reduced, particularly in lipodystrophic women, in both lipomas and perilipoma fat (Fig. 4c). The levels of p53 protein and its target p21 were also reduced in lipodystrophic women (Fig. 4d), but not in the samples from the lipodystrophic man (data not showed).

Discussion

We found no major differences in HE staining, but the adipocytes from lipodystrophic lipomas were bigger than those from other samples. Adipocytes in perilipomatous AT from FPLD2 patients

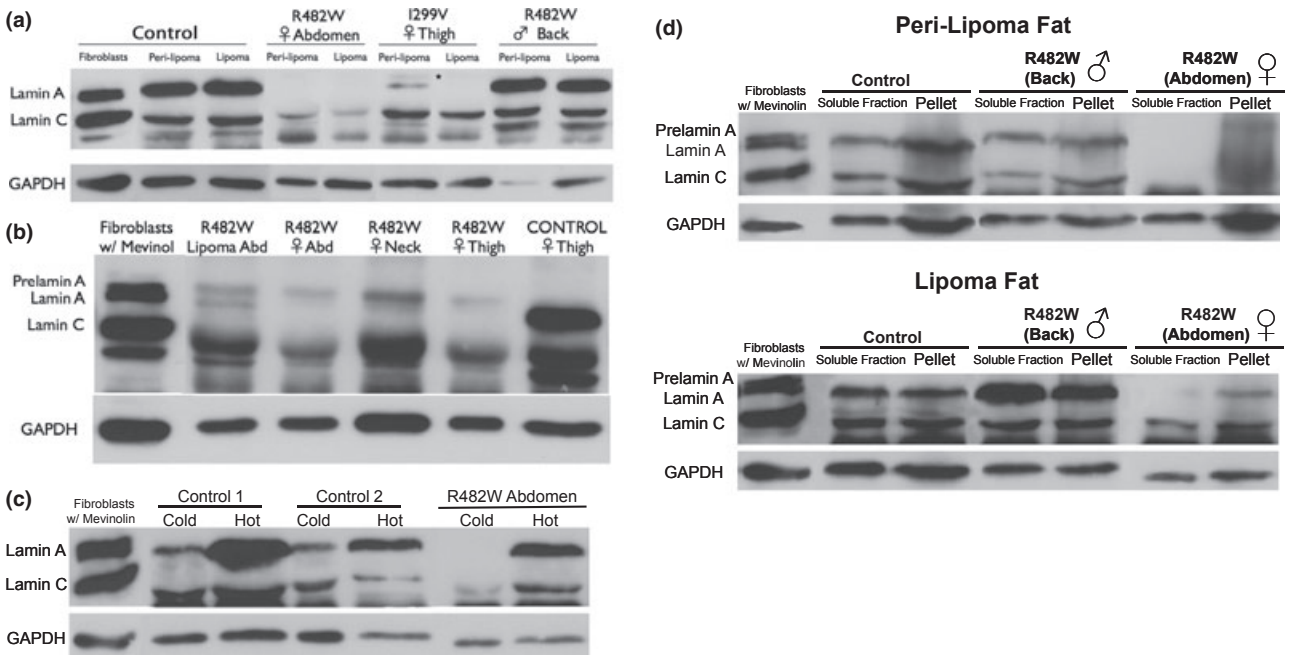

Fig. 3 Detection of prelamin A and lamin A/C by different Western blot protocols in adipose tissue. (a) Western blot analysis of lamin A and C in perilipoma and lipoma fat samples from a representative control and two lipodystrophic patients (bearing R482W and I299V LMNA mutations). *prelamin A band. (b) Western blot analysis of prelamin A, lamin A and lamin C in perilipoma (3rd to 5th lane) and lipoma fat from different anatomical locations of the same type 2 familial partial lipodystrophy (FPLD2) patient (R482W) (6th lane: perilipoma control). Fibroblasts from a control subject were previously treated with mevinolin for 18 h. (c) Western blot analysis of lamin A and lamin C in perilipoma fat from representative controls and a woman with FPLD2 (R482W) using protocols with different temperature and reducing conditions ('hot' and 'cold', see text for explanation). (d) Western blot analysis of lamin A and lamin C in perilipoma and lipoma fat from soluble and insoluble (pellet) protein fractions obtained from a representative control subject, a man with FPLD2 (R482W, lipohypertrophic area, back) and a woman with FPLD2 (R482W, lipoatrophic area, abdomen).

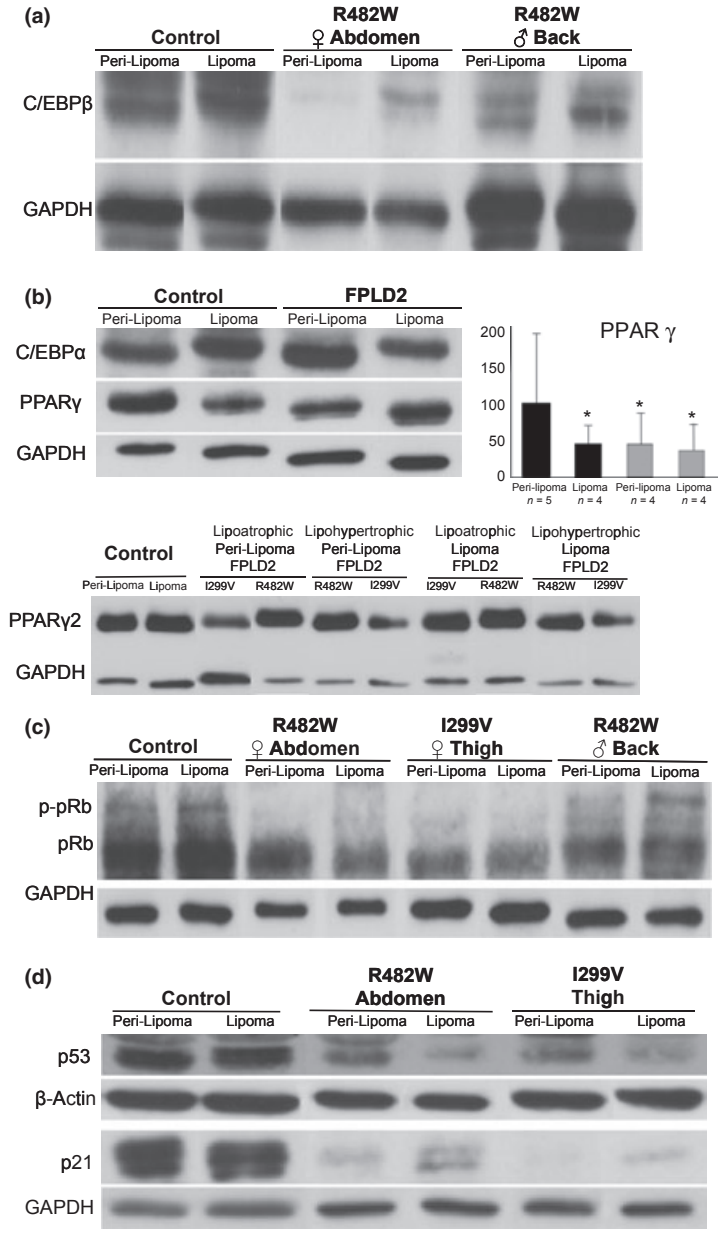

Fig. 4 (a) Western blot analysis of C/EBPβ in perilipoma and lipoma fat samples from a representative control subject and lipodystrophic patients. (b) Western blot analysis of C/EBPα, PPARγ and PPARγ2 and densitometric analysis of PPARγ bands in adipose tissue samples from control subjects (black bars) and type 2 familial partial lipodystrophy (FPLD2) subjects (grey bars). For Western blot analysis $n = 5$ control subjects and $n = 4$ FPLD2 patients. Data are mean ± SD. $^*P < 0{\cdot}05$. (c) Western blot analysis of pRb and phosphorylated pRb (p-pRb) in perilipoma and lipoma fat samples from a representative control and lipodystrophic patients. (d) Western blot analysis of p53 and p21 proteins in perilipoma and lipoma fat samples from a representative control and lipodystrophic patients.

were also larger than those from control subjects. The larger adipocytes in the lipodystrophic AT may seem intriguing, but Wojtanik *et al.* [21] described previously hypertrophic adipocytes in the white AT depots of a FPLD2 transgenic mouse model.

In the light of these histological findings and the lack of UCP1 expression, we can reasonably rule out the hypothesis that the lipomatous AT of FPLD2 patients are associated with a brown fat-like phenotype.

The lipodystrophic AT showed the macrophage and T cell infiltration characteristic of chronic low-grade inflammation, which is associated with insulin resistant obesity,[22] although these findings were not supported by all of the studied inflammatory markers. Even so, the lipodystrophic fat exhibits some features of other disorders that occur with insulin resistance. Increased pro-inflammatory cytokine expression and more macrophages [23] have been found in the subcutaneous AT of patients with HIV-associated lipodystrophy. In a P2-nSREBP-1c transgenic mice, an established model of lipodystrophy, elevated levels of circulating cytokines and infiltrating macrophages have been reported in white AT depots, suggesting systemic inflammation.[24] The behaviour of nonlipody-

strophic lipomas was different and no inflammatory data were found, as noted in a previous study.[11]

Mitochondrial alterations have been reported in cells bearing LMNA mutations,[25] and lipomatosis has been associated with altered mitochondrial DNA because of genetic alterations [10] or mitochondrial disturbances occurring as side effects of antiretroviral treatment.[18] Although the reasons for this dysfunction are unknown, abnormal energy production because of inappropriate expression of components of the respiratory chain or lipid oxidation regulators (e.g. SIRT3) have been hypothesized to elicit a proliferative response in adipose cell depots.

Regarding the genes and proteins involved in adipogenesis, the nonlipomatous and lipomatous AT of lipodystrophic patients showed a decreased expression in practically all of the studied adipogenes, but only in those samples coming from lipoatrophic regions. These results are in accordance with our previous studies.[17]

The process of adipocyte differentiation involves the sequential expression of transcription factors C/EBPβ, C/EBPδ, PPARγ and C/EBPα. Our findings suggest that, despite the important suppression of C/EBPβ and low levels of PPARG and CEBPA transcript expression in the lipodystrophic fat, these transcripts are translated at high enough levels to maintain near-normal levels of the proteins and near-normal phenotype in the scarce AT in these patients.

We found that the major genes involved in the adipogenic process in nonlipodystrophic lipomas were TP53, SREBF1, CEBPA and SLC2A4. The results of gene expression in lipodystrophic lipomas were different, mainly in those coming from lipoatrophic areas in which many adipogenes were severely underexpressed. These findings suggest that other unknown factors are involved in the cell proliferation process of lipodystrophic lipomas, at least in those coming from lipoatrophic areas.

Other authors have investigated the expression of master genes of adipogenesis in nonlipodystrophic lipomas with different results. Guallar *et al.* [10] found lower PPARG expression in lipoma AT, but others [9,11] found no differences. On the other hand, Guallar *et al.* [10] found an increment in CEBPA expression in at least one of the three analysed lipomas.

The putative pathogenetic mechanisms of lipodystrophic laminopathies have been suggested to be related to both a failure of adult stem cell differentiation and prelamin A toxicity, leading to senescence.[26] In the present study, we analysed two major factors in cell cycle control, p53 and pRb. We found that the amount of p53 and its target p21 were dramatically reduced in both lipoma and perilipoma AT from lipodystrophic patients. p53 inhibits adipogenic differentiation of mesenchymal progenitor cells through the downregulation of PPARγ and C/EBPα.[27] Therefore, we would expect to find p53 activation in these fat samples, but our results suggest that the p53 pathway could act as a compensatory mechanism promoting adipogenesis in the scarce AT present in these patients. Yahagi *et al.* [28] found that the p53 and p21 genes were highly induced in the adipocytes of ob/ob mice, leading to the negative regulation of SREBF1 and other lipogenic genes. The authors suggested that the activation of p53 might constitute a negative feedback loop against excess fat accumulation in adipocytes.

Phosphorylated pRb was reduced in lipodystrophic fat samples (lipoma and perilipoma). pRb regulates progression through the cell cycle at the G1 → S-phase transition by inhibiting the activity of E2F-type transcription factors in a phosphorylation-dependent manner.[29] The importance of pRb in adipocyte differentiation is illustrated by the inability of pRb-deficient fibroblasts to undergo adipose conversion[30]; however, the role of pRb in adipogenesis is complex. Dorner *et al.* [31] reported that the LAP2α–lamin A/C complex is critical for pRb-mediated entry of adult stem cells into a postmitotic state, but it is not required for terminal adipocyte differentiation. Whether the decreased p-pRb was a consequence of mutated lamin A/C or that it contributed to less efficient adipocyte differentiation is a question that cannot be answered by our data.

The variability in BMI among FPLD patients is a limitation of the present study. Patients with the distinct LMNA mutations differed moderately in BMI and further analysis with a more refined comparison with controls fully matched for BMI would help to improve the strength of the conclusions.

We found accumulated prelamin A in lipodystrophic fat samples. Accumulation of immature lamin A was reported previously in FPLD2 studies, both *in vivo* and *in vitro*,[16,32] and prelamin A has been involved in impaired adipogenesis in this laminopathy through the sequestration of SREBP1c, a transcription factor implicated in adipose differentiation.[32] Our results suggest that prelamin A accumulation is a necessary, but not sufficient, condition for impaired adipogenesis because this immature protein appears in both lipodystrophic lipoma and lipohypertrophic areas (neck) of a patient with FPLD2. On the other hand, we found different lamin A solubility patterns in the studied fat samples. Other authors [33] have found increased lamin A solubility in R482W fibroblasts, but it was not evaluated in adipocytes. These differences may be cell type specific, which is consistent with the tissue sensitivity of human diseases linked to LMNA mutations. Lamins provide structural support to the interphase nuclear envelope and play a role in its disassembly and reassembly during mitosis. Whether this low solubility plays a role in the pathogenesis of FPLD2 should be addressed in future investigations.

In summary, FPLD2-associated lipomas exhibit similar histological features as nonlipodystrophic lipomas; though, as in nonlipomatous lipodystrophic fat, they show characteristics of chronic low-grade inflammation. In the control lipomas and lipodystrophic lipomas coming from lipohypertrophic areas, TP53, SREBF1, CEBPA and SLC2A4 were the most important factors associated with adipogenesis. The impaired adipogenesis in the lipodystrophic AT seems to be mediated by a malfunction of pRb, C/EBPβ and, probably, PPARγ. Lastly, our results suggest that the p53 pathway may act as a rescue mechanism that allows some adipocyte differentiation in FPLD2 subjects.

Acknowledgements

We are indebted to the patients for their collaboration in this study. This study was supported by the Fundación Mutua Madrileña, PI081449 (Instituto de Salud Carlos III and European Regional Development Fund, FEDER) and PS09/17 (Conselleria de Sanidade, Xunta de Galicia). B.V. is a Research Fellow granted by the Sociedad Española de Lipodistrofias and the Regional Government of Galicia (Xunta de Galicia).

Disclosure

The authors have nothing to disclose.

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Uridine Metabolism in HIV-1-Infected Patients: Effect of Infection, of Antiretroviral Therapy and of HIV-1/ARTAssociated Lipodystrophy Syndrome

Revista: PLoS One. 2010 Nov 15; 5(11):e13896.

PMID: 21085568

Título: Metabolismo de uridina en pacientes infectados por HIV-1: efectos de la infección, terapia antirretroviral y del síndrome lipodistrófico asociado

La Uridina se ha usado en el tratamiento antirretroviral a pesar de que su metabolismo no ha sido completamente comprendido en los pacientes infectados por HIV-1. Las concentraciones en plasma de uridina en los pacientes infectados por HIV-1 eran más bajas que las de los controles. La concentración de uridina en la grasa también resultó ser más baja. Los pacientes con una HALS mixta tenían niveles más altos de uridina en plasma que aquellos que solo presentaban lipodistrofia. La expresión de los genes “uridine cytidine kinase” y “uridine phosphorilase” era significativamente más baja en todos los grupos de pacientes respecto a los controles. Una mayor expresión de los mRNAs para los transportadores de nucleósidos concentrativos fue observada en los pacientes infectados por HIV-1 respecto a los controles. Se concluye que la infección por HIV-1 está asociada con un descenso en los niveles en plasma de uridina y un “shift” de uridina en el tejido adiposo. La terapia antirretroviral no está asociada con las concentraciones de uridina en plasma pero la lipoatrofia que se observa en el síndrome HALS está significativamente asociada con los bajos niveles de uridina en plasma.

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Uridine Metabolism in HIV-1-Infected Patients: Effect of Infection, of Antiretroviral Therapy and of HIV-1/ART-Associated Lipodystrophy Syndrome

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Abstract

Background: Uridine has been advocated for the treatment of HIV-1/HAART-associated lipodystrophy (HALS), although its metabolism in HIV-1-infected patients is poorly understood.

Methods: Plasma uridine concentrations were measured in 35 controls and 221 HIV-1-infected patients and fat uridine in 15 controls and 19 patients. The diagnosis of HALS was performed following the criteria of the Lipodystrophy Severity Grading Scale. Uridine was measured by a binary gradient-elution HPLC method. Analysis of genes encoding uridine metabolizing enzymes in fat was performed with TaqMan RT-PCR.

Results: Median plasma uridine concentrations for HIV-1-infected patients were 3.80 μmol/l (interquartile range: 1.60), and for controls 4.60 μmol/l (IQR: 1.8) (P = 0.0009). In fat, they were of 6.0 (3.67), and 2.8 (4.65) nmol/mg of protein, respectively (P = 0.0118). Patients with a mixed HALS form had a median plasma uridine level of 4.0 (IC95%: 3.40–4.80) whereas in those with isolated lipoatrophy it was 3.25 (2.55–4.15) μmol/l/l (P = 0.0066). The expression of uridine cytidine kinase and uridine phosphorylase genes was significantly decreased in all groups of patients with respect to controls. A higher expression of the mRNAs for concentrative nucleoside transporters was found in HIV-1-infected patients with respect to healthy controls.

Conclusions: HIV-1 infection is associated with a decrease in plasma uridine and a shift of uridine to the adipose tissue compartment. Antiretroviral therapy was not associated with plasma uridine concentrations, but pure lipoatrophic HALS was associated with significantly lower plasma uridine concentrations.

Citation: Domingo P, Torres-Torronteras J, Pomar V, Giralt M, Domingo JC, et al. (2010) Uridine Metabolism in HIV-1-Infected Patients: Effect of Infection, of Antiretroviral Therapy and of HIV-1/ART-Associated Lipodystrophy Syndrome. PLoS ONE 5(11): e13896. doi:10.1371/journal.pone.0013896

Editor: Gary Maartens, University of Cape Town, South Africa

Received June 9, 2010; **Accepted** October 15, 2010; **Published** November 15, 2010

Funding: This work was partially funded by the Fondo de Investigaciones Sanitarias (FIS 02/1280, 05/1591, 07/0976, EC08/00256), the Fundacion para la Prevencion del SIDA en Espana (FIPSE 36610, 36572/06, 36621/06), Ministerio de Ciencia e Innovación (SAF2008-00577), and the Red de Investigacion en SIDA (RIS RD06/006/0022, RD06/0006/1004). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Pyrimidines are synthesized *de novo* through a multistep process starting from glutamine and carbon dioxide to form the pyrimidine ring, orotic acid [1]. The synthesis of orotic acid is catalyzed by dihydroorotate dehydrogenase (DHODH), an enzyme located in the inner mitochondrial membrane, and functional connection to the respiratory chain via ubiquinone ensures efficient oxidation of dihydroorotate [2]. Then, orotate is converted to its nucleotide form in the presence of 5-phosphorylribose-pyrophosphate. Orotate monophosphate is converted by a multifunctional enzyme, uridine monophosphate (UMP) synthase to the nucleotide UMP (Figure 1). UMP is the pivotal nucleotide from which uridine nucleotides di-and triphosphates are formed by ATP-dependent kinases [3]. A large portion of the pyrimidines are salvaged from the degradation of the nucleic acids and nucleotides [4]. The concentration of circulating plasma uridine is tightly regulated throughout different species and individuals [5,6]. The liver appears to have this homeostatic control on uridine degradation and formation [7]. Uridine is essentially cleared in a single pass through the liver and is replaced by "new uridine" formed by *de novo* synthesis [7].

Besides the critical role of uridine in the synthesis of RNA and bio-membranes, there is a role for uridine in regulating a series of biological functions such as complex effects in the regulation of vascular resistance, a role in spermatogenesis, a modulatory effect on the peripheral nervous system, and maintenance of normal CNS activity [8]. In animal models, uridine reduces anxiety, is a

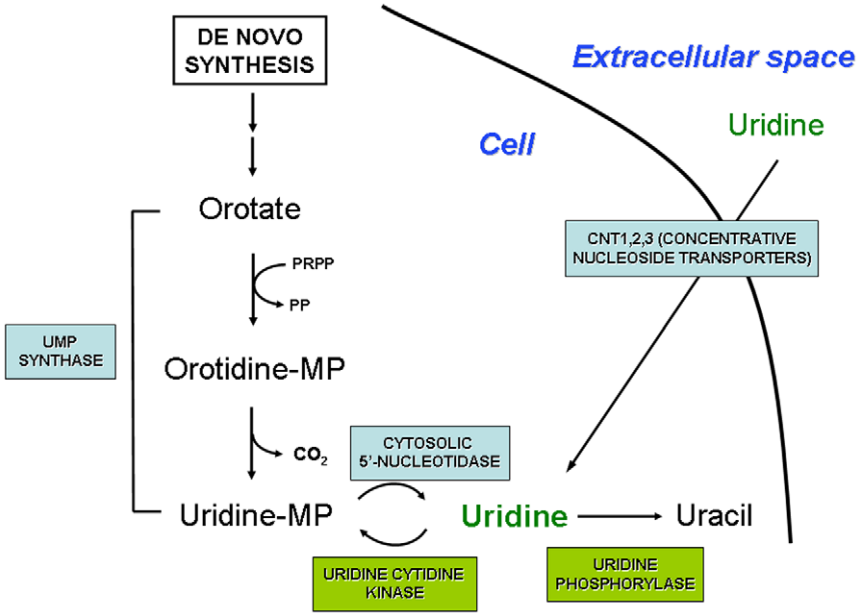

Figure 1. Schematic representation of uridine synthesis and its transport into the cell. MP = monophosphate.
doi:10.1371/journal.pone.0013896.g001

sleep-inducing factor, and may regulate body temperature [8]. Aside from its physiological effects, uridine appears to have remarkable functions in tissues under stress or pathological situations such as restoration of myocardial ATP concentrations [9], maintenance of brain metabolism during ischemia or severe hypoglycemia [10], recovery from neuronal degeneration produced by diabetic neuropathy [11], and as a rescue agent in 5-fluorouracil therapy to decrease bone marrow and gastrointestinal toxicity [12].

The triphosphates are the active forms of the nucleoside analogue reverse transcriptase inhibitors, but they also inhibit polymerase gamma, resulting in mitochondrial DNA depletion [13], although reduction of mitochondrial DNA copy number is also associated to HIV infection in itself [14]. Because the de novo pyrimidine synthesis needs the correct function of the mitochondrial respiratory chain, pyrimidine nucleotide depletion has been hypothesized to contribute to this toxicity by exacerbating the competitive effect of the analogues [15]. However, the effect of these drugs on patients' uridine concentrations has been studied in few cases [16]. Uridine has been able to abrogate the adverse effects of antiretroviral pyrimidine analogues in adipocytes [17], and the benefitial effects of uridine supplementation in cell culture models of stavudine toxicity seems to involve the recovery from nucleoside analogue-induced pyrimidine depletion, because uridine supplementation also prevented the effects of redoxal, a specific DHODH inhibitor which, in turn, exacerbated the mitochondrial toxicity of the stavudine [15]. In a clinical trial, supplementation of uridine to patients on thymidine analogues was associated with fat recovery in limbs [18].

Our working hypothesis was that variations in plasma and fat concentrations of uridine may be involved in the modulation of the toxic effects of thymidine analogues. Specifically, we postulated that the toxic effects of thymidine analogues might result in pyrimidine depletion, detectable as a reduction of systemic and/or fat uridine concentrations. Therefore, the aim of the present study was to look for a relationship between plasma and fat uridine concentrations and HIV-1/highly active antiretroviral (HAART)-associated lipodystrophy syndrome (HALS).

Materials and Methods

Subjects

All patients were recruited through the same clinic at the *Hospital de la Santa Creu I Sant Pau*, which attends a population of 1455 HIV-1-infected patients on active follow-up, and were consecutive patients with an established diagnosis of HIV-1 infection. Patients were eligible whether or not they had HALS, whether or not they were on antiretroviral therapy, and if they were not taking uridine or derivatives. Subjects who were hospitalized or had a frank cognitive impairment such as delirium or dementia on enrolment were not eligible. Patients with opportunistic infections, acute hepatitis, liver insufficiency, neoplasms or fever of undetermined origin were excluded from the study. At the time of study entry no patient used any other drug known to influence glucose metabolism or fat distribution. Informed consent was obtained from the patients at study entry. The diagnosis of AIDS was based on the 1993 revised case definition of the Centers for Disease Control and Prevention (CDC) [19]. Controls were recruited among Hospital personnel and had to be negative for HIV-1 infection and be between 35 and 45 years of age with a proportion of males of about 70%. To be eligible they did not have to meet any of the exclusion criteria. Written informed consent was obtained from patients and controls at study entry. The study was approved by the Ethics Committee of the *Hospital de la Santa Creu i Sant Pau*.

Body composition measurements, definitions of HALS and metabolic syndrome, and biochemistry laboratory measurements

Body composition measurements, definitions of HALS and metabolic syndrome have been described elsewhere [20]. All laboratory investigations were performed as previously described [21]. All laboratory samples, including those for uridine determi-

nation, were obtained after a 12 hr overnight fasting. (Supporting information file S1).

Fat tissue samples

These were obtained from subcutaneous adipose tissue (SAT) depots through a small surgical biopsy performed by an 8 mm punch under local anesthesia with mepivacaine. One half of the SAT obtained was immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction (see below). The remaining was used in fresh for determining fat uridine concentrations.

Measurement of plasma and fat uridine concentrations

For uridine determination, anticoagulated blood was centrifuged at 1,500× g and plasma was separated and kept at −20 C until analysis. Fat tissue was homogenated in the presence of 10 volumes (e.g., 50 mg of tissue +500 µl) of homogenization buffer (10 mmol/l HEPES pH 7.5; 5 mmol/l EDTA; 5 mmol/l DTT; 5 mmol/l $MgCl_2$; plus *Complete-Mini*^{TM} protease inhibitor cocktail, Roche), in an ice-cold bath. After centrifugation, the supernatant was saved and protein concentration was determined by the Bradford method [22].

Uridine was measured by a binary gradient-elution HPLC method. 200 µl of plasma or tissue homogenate were deproteinized by the addition of 9 µl of cold perchloric acid 11.7 mol/l (final concentration 0.5 mol/l) and kept 5 min on ice. Precipitated proteins were eliminated by centrifugation and 5 µl of the supernatant were injected into an Acquity UPLC apparatus (Waters, Milford, MA) and eluted at 0.5 ml/min with a saline buffer eluent A (20 mM ammonium acetate, pH 5.6) and an organic eluent B (methanol gradient grade) according to the following gradient: 0 to 1.1 min, 100% eluent A; 1.1 to 6.1 min, 100% to 82.6% eluent A; 6.1 to 6.2 min, 82.6 to 100% eluent A; 6.2 to 7.2 min, 100% eluent A. The column used was an Acquity UPLC BEH C18 column 100×2.1 mm, 130 Å pore size, 1.7 µm particle size (Waters). Optical absorbance of the eluate was monitored at 267 nm and definitive identification of the uridine peak was based upon retention time and treatment of a second aliquot of the sample with a large excess of purified *E. coli* thymidine phosphorylase (Sigma-Aldrich, St. Louis, MO) to eliminate enzymatically the uridine peak. The area from residual peaks coeluting with uridine and still present after treatment with *E. coli* thymidine phosphorylase was substracted from the area of the uridine peak obtained in the untreated aliquot, to ensure no overestimation of uridine concentration. The quantitation of the nucleoside was based on peak areas using external aqueous standards. The method had a between-day imprecision of 2.3% (coefficient of variation) for a uridine concentration of 4.30 µmol/l. Uridine concentrations in plasma of ~3 to 5 µmol/l are found in different species and individuals [5,6,12].

Expression of genes encoding uridine metabolism-related enzymes and nucleoside transporters

RNA extraction was performed using a column-affinity based methodology (Rneasy, Qiagen, Hilden. Germany). On-column DNA digestion was performed during RNA purification (Rnase-Free Dnase set, Qiagen). TaqMan Reverse Transcription and RT-PCR reagents were used for mRNA analysis (Applied Biosystems, Foster City, USA). One microgram RNA was transcribed into cDNA random-hexamer primers and the real-time reverse transcriptase-polymerase chain reaction was performed on the ABI PRISM 7700HT sequence detection system (Applied Biosystems). The TaqMan RT-PCR reaction was performed in a final volume of 25 µl using TaqMan Universal PCR Master Mix, NoAmpErase UNG reagent and the specific gene expression primer probes. The TaqManGene Expression assays (Applied Biosystems) used were: uridine-monophosphate synthase, Hs00923516; uridine cytidine kinase-1, Hs00258815; uridine phosphorylase, Hs00427695; uridine 5′-monophosphate hydrolase (also called cytosolic 5′-nucleotidase III), Hs00826433; concentrative nucleoside transport (CNT-1), Hs00984402; CNT-2, Hs01035852; CNT-3, Hs00223220. Controls with no RNA, primers, or reverse transcriptase were included in each set of experiments. Each sample was run in duplicate and the amount of the mRNA for the gene of interest in each sample was normalized to that of the reference housekeeping control (HPRT1, Hs999999009), as already reported [23] using the comparative (2-ΔCT) method. Calculations based on a second, independent, housekeeping gene (18S rRNA) led to similar results. Data are expressed as means ± SEM.

Measurement of plasma fatty acid concentrations

The serum composition of fatty acids was determined using the method by Lepage and Roy [24]. Aliquots of 300 µL of plasma were transferred into glass tubes for direct transesterification. 2 mL of methanol-benzene (4:1, v/v) with internal standard (heptadecanoic acid, C17:0) and 0.01% butylhydroxytoluene, as antioxidant. Samples were vortexed at low speed while slowly adding 200 µL of acetyl chloride, little by little, over a period of 2 minutes. The tubes were tightly closed with teflon-lined caps and vortexed 30 seconds.

Samples were then heated for 60 minutes at 100°C in a heating block and shaking continuously at 600 rpm. After the tubes had been cooled to room temperature, five millilitres of 6% (w/v) potassium carbonate were then added. The samples were vortexed for 30 seconds and centrifuged at 2500 rpm for 20 minutes at 15°C. The fatty acid methyl esters contained in the upper benzene phase were transferred to gas chromatography vials and stored at 4°C until injection into the chromatograph.

The analysis was performed on a Varian CP-3900 gas chromatograph equipped with a flame ionization detector, using a capillary column model CP9205-VF-WAXms (Varian), 30 m length ×0.25 mm internal diameter ×0.25 µm film thickness. Individual fatty acids were identified by order of elution and upon comparison with known commercially prepared fatty acid standards (GLC 566-C, Nu-Chek Prep Inc.). Fatty acid methyl ester peaks were identified by comparison of retention times of standards and quantified in comparison to known commercially prepared reference standards. The percentage of each fatty acid class was expressed as percentage of total fatty acids.

Statistical analyses

All analyses were performed with the Statistical Package for Social Sciences version 17.0 (SPSS, Chicago, IL). Data are expressed as mean ± SD or median with interquartile range (IQR). Data that were not normally distributed, as determined using the Kolmogorov-Smirnov test, were logarithmically transformed before analysis. Student's t test was used for comparison between two groups, Pearson's correlations or one-way ANOVA and multiple testing were corrected using Bonferroni correction. Stepwise logistic regression analysis was used to examine the association of plasma uridine concentrations and other parameters with HALS. The variables selected to enter into stepwise regression were those that correlated significantly with plasma uridine concentrations (after Bonferroni correction for multiple testing). In all statistical tests, P values <0.05 were considered significant.

Results

Population studied

Two-hundred and twenty one HIV-1-infected patients and 35 healthy controls were studied. The demographics, HIV-1 infection, and antiretroviral exposure parameters from patients and controls are shown in table 1. Mean duration of HIV-1 infection was 7.8±6.8 years (median: 7.0 [IQR: 12.2 years]), and 63 patients (28.5%) had had a prior AIDS-defining condition. Fifty-nine patients were co-infected with hepatitis C virus (26.7%), whereas 10 (4.5%) had chronic hepatitis B virus infection. Anthropometric, metabolic data and plasma uridine concentrations are shown in Table 2.

Antiretroviral drug exposure and immuno-virological situation

Among treated patients, 59 (41.3%) had undetectable viral load at the moment of the study. The other 84 treated patients presented a median detectable viral load of 2.3 $\log_{10}$ copies/ml (IQR: 1.1 $\log_{10}$ copies/ml). The mean CD4 count was 589±320 cells/mm^3 (median: 531 [IQR. 425] cells/mm^3). Nadir CD4 cell count was <100 cells/mm^3 in 57 patients (25.8%). The cumulated exposure to antiretroviral drugs is shown in table 1. Twenty patients (13.9%) of the treated group were on an antiretroviral regime that included AZT whereas 25 (17.5%) were on a d4T-based regime at the moment plasma and fat uridine concentrations were measured. There were not statistically significant differences in plasma and fat uridine concentrations between patients currently on AZT (P = 0.9930) or d4T (P = 0.4944), either considered individually or as a combined group (P = 0.5809).

Table 1. Demographics, HIV-1 infection and antiretroviral exposure parameters.

Parameter	Controls (n = 35)	HIV-1-infected patients			P value
		Naïve (n = 78)	HALS- (n = 59)	HALS+ (n = 84)	
Age, mean ± SD, (y)	43.7±5.0	36.9±10.2	44.1±8.0	46.2±8.9	< 0.0001
Sex, men (%)	25 (71.4)	67 (85.9)	46 (77.9)	60 (71.4)	0.1302
Years of infection	----	2.0 (1.0)	11.0 (10.0)	13.0 (6.0)	< 0.0001
AIDS (%)	----	3 (3.9)	18 (30.6	42 (50.0)	< 0.0001
HCV infection (%)	----	7 (9.5)	16 (27.6)	36 (42.9)	< 0.0001
CD4 count/mm^3	----	431 (302)	566 (416)	50 (463)	0.0136
Plasma viral load ($\log_{10}$)	----	4.3 (1.2)	1.8 (1.1)	1.7 (1.5)	< 0.0001
Antiretroviral drug exposure					
NRTI (m)	----	----	190.0 (106.0)	225.0 (83.0)	0.0069
NNRTI (m)	----	----	35.0 (52.7)	49.0 (48.5)	0.0519
PI (m)	----	----	43.0 (51.2)	49.0 (48.0	0.1114
AZT (m)	----	----	22.0 (71.0)	20.0 (47.7)	0.5533
AZT (g)	----	----	297.0 (852.0)	240.0 (824.5)	0.7448
d4T (m)	----	----	25.0 (61.2)	64.0 (37.5)	< 0.0001
d4T (g)	----	----	62.4 (138.9)	144.9 (89.7)	< 0.0001
3TC/FTC (m)	----	----	50.0 (77.0)	52.0 (65.5)	0.5220
ddI (m)	----	----	10.5 (33.5)	35.5 (61.5)	0.0028
ABC (m)	----	----	0(0)	0 (14.0)	0.8825
TDF (m)	----	----	10.0 (35.0)	8.0 (34.5)	0.6102
EFV (m)	----	----	0 (35.0)	2.5 (44.0)	0.2175
NVP (m)	----	----	0 (33.5)	6.0 (46.0)	0.1400

Parameters are expressed as median (interquartile range) unless specified. HCV, hepatitis C virus, NRTI = nucleoside-analogue reverse transcriptase inhibitor, NNRTI = non nucleoside-analogue reverse transcriptase inhibitor; PI = protease inhibitor; AZT = zidovudine, d4T = stavudine, 3TC = lamivudine, FTC = emtricitabine, ddI = didanosine, ABC = abacavir, TDF = tenofovir; EFV = efavirenz, NVP = nevirapine, m = months; g = grams. HALS = HIV-1/HAART-associated lipodystrophy syndrome.

doi:10.1371/journal.pone.0013896.t001

Diagnosis of HALS and metabolic syndrome

HALS, based on 7 or more points on the Lipodystrophy Scale Grading Score (LSGS), was diagnosed in 84 treated patients (58.7%) whereas absence of HALS was found in 59 treated patients (41.3%), and in all the naives. All 84 patients presented with lipoatrophy, but in addition 44 (52.4%) had a mixed lipoatrophic-lipohypertrophic form. Differences between HALS and non-HALS patients are shown in tables 1 and 2. Patients with a mixed form had a median plasma uridine level of 4.0 (IC95%: 3.40–4.80) whereas in those with isolated lipoatrophy it was 3.25 (2.55–4.15) µmol/l/l (P = 0.0066).

Plasma uridine concentrations and its correlation with cardio-metabolic, HIV-1 infection and antiretroviral therapy factors

Plasma uridine concentrations for HIV-1-infected patients ranged from 1.60 to 7.4 µmol/l/l, with a mean value of 3.85±1.18 µmol/l (median: 3.80; IQR: 1.60). The corresponding values for controls were: range: 2.0–9.4 µmol/l, mean: 4.36±1.28 µmol/l, median: 4.60, IQR: 1.80 µmol/l (P = 0.0009). Plasma uridine concentrations were not significantly different between HALS and non-HALS patients (P = 0.3377). Patients with plasma uridine concentrations <3 µmol/l were 8.9% among controls, 16.7% among naïves, 19.0% among treated patients without HALS, and 29.6% among patients with HALS (P = 0.0460). There was no sex difference in plasma uridine concentrations for HIV-1-infected patients, but there were statistically significant differences between controls (men: median 5.0 [IQR: 1.5] vs. women: 3.45 [1.4], P = 0.0262). In naïve patients, there was a negative correlation between plasma concentrations of uridine and viral load (R = −0.2580, P = 0.0228). Patients who were currently receiving treatment with AZT or d4T had a median plasma uridine level of 3.90 [IQR: 1.67] whereas in those who did not it was 3.70 [IQR: 1.80) µmol/l (P = 0.4278). There was no correlation between cumulated d4T use either in time (months) (R = 0.094, P = 0.3935) or grams (R = 0.078, P = 0.4815). There were not statistically significant differences between patients who did or did not have AIDS, CD4 nadir <100 cells/mm^3, did or did not have not clinical toxicity (peripheral neuropathy or pancreatitis), HALS, HCV infection, and metabolic syndrome with respect to plasma uridine concentrations. Lean subjects had median plasma uridine concentrations of 3.7 [IQR: 1.5], overweight subjects 4.1 [IQR: 1.8], and obese subjects 3.9 [IQR: 1.67] µmol/l (P = 0.0152 for lean vs. obese). The correlations of plasma uridine concentrations with metabolic, infectious and treatment factors are shown in table 3. Plasma uridine concentrations were not found to be independently associated either with the metabolic syndrome or with HALS in HIV-1-infected patients.

Table 2. Anthropometric, metabolic data and plasma uridine levels.

Parameter	Controls (n = 35)	HIV-1-infected patients			P value
		Naïve (n = 78)	HALS- (n = 59)	HALS+ (n = 84)	
BMI	24.4 (2.7)	23.6 (4,6)	23.8 (5.2)	23.5 (4.7)	0.1307
Waist circumference (cm)	89.0 (13.2)	86.0 (14.0)	88.0 (13.0)	86.5 (15.5)	0.0055
Waist-to-hip ratio	0.88 (0.09)	0.91 (0.11)	0.93 (0.09)	0.93 (0.11)	< 0.0001
Total body fat (%)	24.8 (5.8)	21.5 (9.9)	22.5 (10,2)	19.7 (10.5)	< 0.0001
Trunk/apendicular fat ratio	1.15 (0,5)	1.4 (1.2)	1.4 (1.1)	2.4 (1.2)	< 0.0001
Total cholesterol (mmol/l)	5.2 (1.4)	4.7 (1.5)	4.9 (1.0)	4.8 (1.7)	0.0003
Triglycerides (mmol/l)	0.82 (0.43)	1.5 (1.4)	1.9 (1.3)	2.0 (1.7)	< 0.0001
HDL cholesterol (mmol/l)	1.5 (0.5)	1.2 (0.5)	1.2 (0.5)	1.2 (0.4)	< 0.0001
LDL cholesterol (mmol/l)	3.3 (1.3)	2.7 (1.2)	2.5 (1.0)	2.8 (1.0))	0.0027
MUFAs (% with respect to total fatty acids)	23.9 (4.3)	26.3 (5.0)	27.9 (6.6)	28.3 (5.9)	< 0.0001
PUFAs (% with respect to total fatty acids)	44.7 (4.0)	40.4 (5.5)	38.7 (7.3)	37.2 (7.6)	< 0.0001
Glucose (mmol/l)	4.8 (0.6)	5.1 (0,9)	5.3 (0,7)	5.5. (1.1)	< 0.0001
Insulin (pmol/l)	26.0 (33.2)	48.5 (63.5)	48.0 (54.2)	85.5 (61.5)	< 0.0001
HOMA-IR	0.5 (0.7)	0.9 (1.1)	0.9 (1.0)	1.6 (1.2)	< 0.0001
Systolic BP (mm Hg)	118.0 (17.7)	120 (16.5)	120 (20.0)	120 (20.0)	0.2649
Diastolic BP (mm Hg)	69.0 (15.0)	75.0 (10.0)	78 (12.0)	78 (10.0)	0.0081
Metabolic syndrome (%)	5 (14.3)	5 (6.4)	18 (30.5)	28 (33.3)	< 0.0001
Uridine (μmol/l)	4.6 (1.8)	3.8 (1.3)	3.8 (1.6)	3.7 (1.7)	0.0034

Parameters are expressed as median (interquartile range) unless specified. P-values between individual groups are shown when <0.05. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein, HOMA-IR, homeostasis model assessment of insulin resistance; HALS = HIV-1/HAART-associated lipodystrophy syndrome.

doi:10.1371/journal.pone.0013896.t002

Fat uridine concentrations

Uridine content was measured in fat biopsy samples from 19 patients (naïve: 10, no HALS: 5, HALS: 4) and 15 controls. Median uridine level in fat from HIV-1-infected patients was 6.0 [IQR: 3.67], and in controls, 2.8 [4.65] nmol/mg protein ($P = 0.0118$). Median fat uridine level was 6.1 [0.50], 2.6 [1.85], and 6.3 [3.4] nmol/mg protein, for naïve, non-HALS patients, and HALS patients, respectively ($P = 0.3767$). There was a negative correlation between plasma and fat uridine concentrations in HIV-1-infected patients ($R = -0.5210$, $P = 0.0223$), whereas such a correlation was not found for controls ($R = 0.0680$, $P = 0.8720$). Median fat uridine level was 5.5 [IQR: 4.0] nmol/mg protein for those who were currently treated with AZT or d4T, whereas it was 2.6 [IQR: 1.27] nmol/mg protein for those not treated ($P = 0.5369$). There were no statistically significant differences between patients with mixed HALS and pure lipoatrophic HALS with respect to fat uridine concentrations 6.80 [IQR: 0] vs. 6.55 [IQR: 0.50] nmol/mg protein, respectively, $P = 0.4028$). The only correlations were for EFV exposure ($R = -0.4890$, $P = 0.0336$), and for PUFAs level ($R = -0.5740$, $P = 0.0252$).

Expression of genes encoding uridine metabolism-related enzymes and concentrative uridine transporters

The expression of genes involved in uridine metabolism is depicted in figure 2. The expression of the transcript for UMP synthase was not significantly modified in adipose tissue from all HIV-1 infected patient groups with respect to controls, regardless of having been treated or having developed HALS or not. The expression of the gene for UMP hydrolase (also called cytosolic 5′-nucleotidase III) was slightly decreased in naïve patients but unaltered in the other groups of HIV-infected patients (Figure 2). The pattern of changes observed for uridine cytidine kinase and uridine phosphorylase (UPase) indicated a significant decrease in all groups of patients with respect to controls. There were no changes in comparisons between naïve and treated patients or in patients with or without HALS. The expression of concentrative nucleoside transporters (CNTs), potentially involved in concentrating intracellular uridine [25], showed a similar pattern of changes in the three subtypes of transporters in naïve and treated patients, in agreement with previous findings [23]. Naïve patients showed a higher expression of the mRNAs for CNTs with respect to healthy controls, which was statistically significant for CNT1 and CNT3. HAART-treated patients, either having HALS or not, showed significantly higher concentrations of expression of the three CNT mRNA subtypes with respect to controls and naïve, with distinct concentrations of statistical significance depending on the CNT subtype (Figure 2). No differences were observed for the expression of CNTs mRNAs between patients with or without HALS.

Discussion

Our study does not suggest an association of the development of features of HALS or metabolic syndrome with plasma and fat concentrations of uridine, except for lipohypertrophy, but suggests that HIV-1 infection is associated with a significant decrease in uridine plasma concentrations. However, the number of patients with uridine <3 μmol/l was higher in patients with than in non-HALS patients. Antiretroviral therapy that included thymidine

Table 3. Correlations of serum uridine levels with metabolic, infectious and treatment factors.

	Serum uridine (μmol/l)		Serum uridine (BMI-adjusted)	
	r	P	r	P
BMI	0.1280	0.0465		
Age	0.0140	0.8205	−0.0040	0.9470
Waist circumference	0.0970	0.1218	0.0350	0.5890
WHR	−0.0010	0.9868	−0.0440	0.4920
Fat percentage	0.1630	0.0093	0.1250	0.0520
Trunk/apendicular fat ratio	− 0.0630	0.3126	-0.0690	0.2830
Fasting glucose	−0.0820	0.1950	-0.0990	0.1260
Fasting insulin	0.0420	0.5092	−0.0650	0.3170
HOMA-IR	−0.0550	0.3785	−0.0790	0.2200
MUFAs	−0.2830	< 0.0001	-0.2900	< 0.0001
PUFAs	0.3130	< 0.0001	0.3220	< 0.0001
Total cholesterol	0.2010	0.0010	0.1700	0.0080
Triglycerides	−0.0210	0.7434	−0.0490	0.4490
LDL cholesterol	0.1950	0.0019	0.1650	0.0110
HDL cholesterol	0.1440	0.0217	0.1650	0.0100
VLDL cholesterol	−0.0460	0.4703	−0.0790	0.2260
Systolic BP	0.0370	0.5550	0.0370	0.5680
Diastolic BP	0.0020	0.9775	−0.0050	0.9330
CD4 count	0.0200	0.7723	0.0150	0.8350
Plasma viral load (log_{10})	−0.0120	0.8640	0.0050	0.9470
Years of infection	−0.2230	0.0005	−0.2130	0.0010
NRTI (m)	−0.1670	0.0085	−0.1710	0.0080
NNRTI (m)	−0.0510	0.5506	−0.0300	0.7330
PI (m)	−0.1090	0.0864	−0.1130	0.0800
AZT exposure (m)	−0.0300	0.6352	−0.0220	0.7340
AZT exposure (g)	−0.0530	0.4058	−0.0480	0.4620
d4T exposure (m)	−0.1700	0.0074	−0.1760	0.0070
d4T exposure (g)	−0.1640	0.0097	−0.1720	0.0080
ddI exposure (m)	−0.1280	0.0437	−0.1190	0.0670
EFV exposure (m)	−0.1490	0.0182	−0.1500	0.0200
NVP exposure (m)	−0.0410	0.5164	−0.0410	0.5320

BMI = body mass index, WHR = waist-to-hip ratio, HOMA-r, homeostasis model assessment of insulin resistance, MUFAs = monounsaturated fatty acids, PUFAs = polyunsaturated fatty acids, LDL = low density lipoproteín, HDL = high density lipoproteín, VLDL = very low density lipoproteín, BP = blood pressure, NRTI = nucleoside-analogue reverse transcriptase inhibitor, NNRTI = non nucleoside-analogue reverse transcriptase inhibitor; PI = protease inhibitor; AZT = zidovudine, d4T = stavudine, ddI = didanosine, EFV = efavirenz, NVP = nevirapine, m = months; g = grams.

doi:10.1371/journal.pone.0013896.t003

analogues did not influence uridine plasma concentrations. On the other hand, fat uridine concentrations showed an inverse correlation with plasma concentrations. This finding may be explained by an over-expression of those genes that encode concentrative nucleoside transporters, and particularly CNT3 [23,25], as well as by under-expression of genes encoding enzymes leading to anabolic (UCK) or catabolic (UPase) uridine consumption.

However, the present results have to be viewed in the light of their inherent limitations. The first one comes from the nature of the study; this is a cross-sectional study and therefore, no causal relationships can or should be drawn. Second, for the majority of patients, uridine was measured in plasma whereas the anatomical and biological processes giving rise to HALS occur in the adipose depots. We have some measurements of uridine in fat, and although such measurements are highly reproducible and with tight variability coefficients, their reliability is difficult to ascertain [26]. In addition, the number of patients included in this substudy was low, and this may hamper the validity of our results.

The plasma uridine results are trustworthy since we were especially careful to avoid overestimations of the uridine peak, as we have observed that some unknown compounds sometimes coelute with uridine in our chromatographic separation. This fact could explain, at least in part, the values slightly higher for uridine plasma concentration reported by other groups [18], and could account for the difference between our results, which do not detect reduced uridine concentrations in treated patients, an the work by Langmann et al that, in contrast, reports significat reductions in dideoxy-NRTI treated patients [16]. We double tested enzymatically the peak of uridine by treating a second aliquot of the

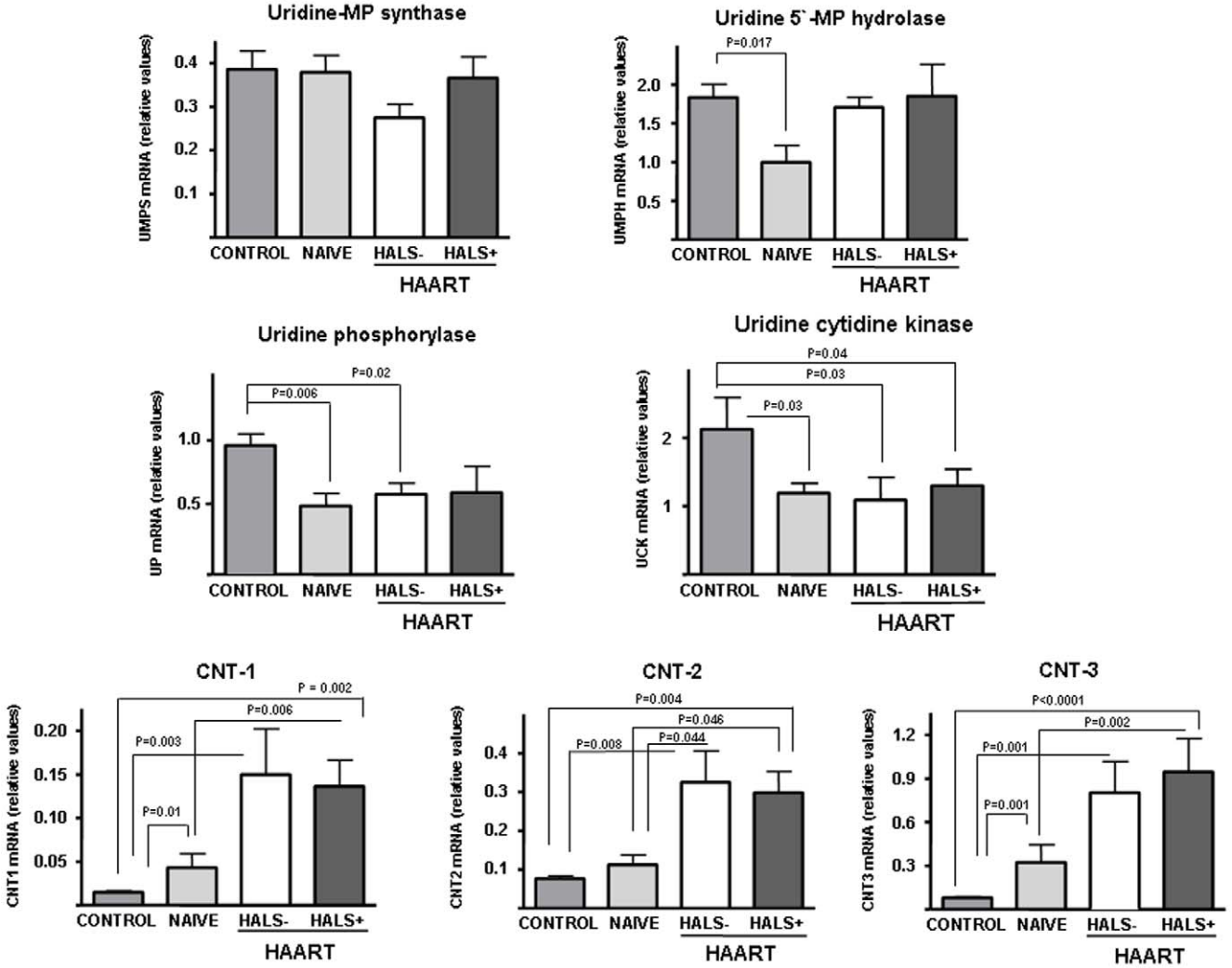

Figure 2. Expression of genes encoding enzymes involved in uridine metabolism and concentrative nucleoside transporters (CNT). Data from subcutaneous adipose tissue of uninfected controls and HIV-1 infected patients. Figure shows means + SEM for each specific mRNA concentration (expressed as ratio relative to HPRT mRNA) from 6-8 patients/group. P values for statistical comparisons between groups are shown when <0.05. MP = monophosphate. HALS- = No HIV-1/HAART-associated lipodystrophy syndrome. HALS+ = HIV-1/HAART-associated lipodystrophy syndrome. HAART = Highly Active AntiRetroviral Therapy.
doi:10.1371/journal.pone.0013896.g002

samples with an excess of thymidine phosphorylase from *E. coli*, which ensures that we only measured true uridine, with no interfering compounds eluting at the same retention time in the chromatogram. This may explain why plasma uridine values obtained in our patients with HALS are lower than those reported by Sutinen et al. [18] for patients with clinically evident lipoatrophy. On the other hand, the results obtained in our study compared well with previously reported uridine plasma concentrations [5,6,12].

We have found that HIV-1 infection is associated with significantly lower concentrations of uridine in plasma, whereas there were not differences between HIV-1-infected patients irrespective of their treatment status, but it was by the presence of lipoatrophic HALS. This may be explained by the fact that, unlike what happens in non-infected patients, there is a shift of uridine towards adipose depots and maybe other tissues. Additionally, among naïve patients, a negative correlation was found between uridine plasma concentrations and the amount of plasma HIV-1 RNA. This may suggest its consumption during the HIV-1 replicative cycle. On the other hand, alterations due to HIV-1-infection in adipose tissue from patients, i.e. reduction in the amounts of mitochondrial DNA [27], or impaired expression of genes implicated in adipocyte anabolic functions [28], have been previously reported.

Uridine supplementation has been advocated as a successful treatment for thymidine analogues-related lipoatrophy based on a short-term randomized, double-blind, placebo-controlled trial [18]. However, recent data from a randomized, double blind, placebo-controlled trial, failed to show any benefit of uridine supplementation in terms of fat gain after 48 weeks of treatment [29]. In addition, when compared with a strategy of switching from thymidine analogues to tenofovir, supplementation with uridine induced a pro-inflammatory state without showing benefit in terms of fat gain [30]. Therefore, it seems that uridine administration cannot reverse the adipose toxic effects of thymidine analogues, and may even represent an additional risk because of its potential inflammatory induction. This is not at odds with our findings concerning the positive correlation between

plasma uridine concentrations and polyunsaturated fatty acid (PUFAs) concentrations. The beneficial effects of PUFAs on the cardiovascular system and on lipid profile are well known [31].

The concentrations of uridine in animal experimentation tissues are usually far in excess of the concentration of uridine in plasma [1]. Both, plasma and intracellular concentrations of uridine are regulated by the catabolic activity of uridine phosphorylase (UPase) and by transport mechanisms [9–11,32]. We have found an underexpression of UPase in adipocytes which may partly justify the greater concentration of fat uridine compared to that of plasma (Figure 2). In addition, the increased expression of CNTs in HIV-1-infected patients' adipose tissue contribute to the shift of uridine into the adipose cells. The overall shift in the expression of these genes leading to induce an increase in the fat tissue pyrimidine pool fits well with a previous report suggesting a role of intracellular pyrimidine depletion associated to nucleoside analogues toxicity [15]. Although we did not see any effects in the systemic uridine concentrations, the increase of intracellular uridine in treated patients might be, in fact, a reflex of a compensatory mechanism to avoid intracellular pyrimidine depletion.

In summary, our results suggest that uridine homeostasis is profoundly disturbed by HIV-1 infection. Although plasma concentrations in HIV-1-infected patients were lower than in controls, the only additional uridine disturbance that could be documented was unrelated to antiretroviral therapy but it was to the development of pure lipoatrophic syndrome. These results are in agreement with the lack of efficacy of uridine supplements in HALS reversal as shown by ACTG 5229.

Supporting Information

File S1

Found at: doi:10.1371/journal.pone.0013896.s001 (0.03 MB DOC)

Author Contributions

Conceived and designed the experiments: PD ALA RM. Performed the experiments: JTT MG JCD JMGE PCS IF MPA F. Villarroya ALA RM. Analyzed the data: PD JTT VP MG JCD MdMG MGM IF MPA F. Vidal F. Villarroya ALA RM. Contributed reagents/materials/analysis tools: JTT MG JCD MdMG JMGE MGM PCS MPA F. Villarroya ALA. Wrote the paper: PD F. Vidal F. Villarroya ALA RM. Selected and recruited patients: VP MdMG MGM IF.

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Adipogenic/Lipid, Inflammatory, and Mitochondrial Parameters in Subcutaneous Adipose Tissue of Untreated HIV-1–Infected Long-Term Nonprogressors: Significant Alterations Despite Low Viral Burden

Revista: Journal of Acquired Immune Deficiency Syndrome. 2012 May 10. [Epub ahead of print]

PMID: 22580565

Título: Parámetros adipogénicos, lipídicos, inflamatorios y mitocondriales en el tejido adiposo de pacientes infectados por HIV-1 LTNPs ("Long Term Nonprogressors"): Alteraciones significativas a pesar de una baja carga viral

La infección por HIV-1 puede inducir alteraciones en el tejido adiposo de los pacientes infectados por la vía de las proteínas del virus y la inflamación. No se conoce si este hecho ocurre en los pacientes infectados por HIV-1 LTNP ("Long Term Nonprogressors"). El objetivo de este estudio es determinar si los parámetros de diferenciación adipocitaria, lípidos, inflamación y mitocondriales están alterados en el tejido adiposo subcutáneo abdominal de dichos pacientes. En cuanto a los parámetros adipogénicos y lipídicos, la expresión de PPARγ, LPL y FABP-4 aparecen disminuidos en los pacientes respecto a los controles. Por otro lado la expresión de PPARγ era mayor en LTNPs que en pacientes típicos aún no tratados. En cuanto a parámetros inflamatorios la expresión de TNFα, IL-18 y b2-MCG era significativamente mayor en LTNPs que en controles sanos. Por otro lado la expresión de IL-18 era mayor en pacientes típicos aún no tratados que en LTNPs. En lo que a parámetros mitocondriales se refiere el DNA mitocondrial aparece significativamente reducido en LTNPs comparado con pacientes típicos aún no tratados y controles sanos. La expresión de COII y COIV también aparece disminuida en LTNPs comparado con pacientes típicos aún no tratados y controles sanos. Se puede concluir que el tejido adiposo de los LTNPs presenta trastornos significativos en algunos marcadores adipogénicos así como procesos inflamatorios, pero en un grado menor a lo observado en los pacientes típicos aún no tratados.

Adipogenic/Lipid, Inflammatory, and Mitochondrial Parameters in Subcutaneous Adipose Tissue of Untreated HIV-1–Infected Long-Term Nonprogressors: Significant Alterations Despite Low Viral Burden

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Background: HIV-1 can induce disturbances in adipose tissue in infected subjects through the effects of some of its proteins or inflammation. It is not known whether this also takes place in HIV-1–infected long-term nonprogressors (LTNPs). Our objectives were to determine whether adipocyte differentiation/lipid, inflammatory, and mitochondrial parameters are perturbed in abdominal wall subcutaneous adipose tissue of untreated HIV-1–infected patients LTNPs.

Methods: Cross-sectional study involving 10 LTNPs, 10 typical progressors (TPs), and 10 uninfected controls (UCs). The parameters assessed were peroxisome proliferator–activated receptor-gamma (PPARγ), lipoprotein lipase, and fatty acid–binding protein 4 mRNA (adipogenic/lipid); tumor necrosis factor-alpha, interleukin 18 (IL-18), β2-MCG, monocyte chemoattractant protein 1, CD1A, and C3 mRNA (inflammation); and cytochrome c oxidase subunit II (COII), COIV, CYCA, nuclear respiratory factor 1, PPARγ coactivator 1α mRNA, and mtDNA content (mitochondrial).

Results: Regarding adipogenic/lipid parameters, LTNPs had PPARγ, lipoprotein lipase, and fatty acid–binding protein 4 mRNA significantly decreased compared with UCs ($P \leq 0.001$ for all comparisons). PPARγ mRNA was significantly greater in LTNP than in TP ($P = 0.006$). With respect to inflammatory parameters, tumor necrosis factor-alpha, IL-18, and β2-MCG mRNA were significantly higher in LTNPs compared with UCs ($P < 0.005$ for all comparisons), whereas IL-18 mRNA was greater in TPs compared with LTNPs ($P = 0.01$). As mitochondrial parameters are concerned, mtDNA was significantly reduced in LTNPs compared with TPs ($P = 0.04$) and UCs ($P = 0.03$). COII and COIV were also significantly reduced in LTNPs compared with UCs and TPs.

Conclusions: Adipose tissue from untreated LTNPs may have limited but significant derangements in some adipogenic/lipid and may have inflammatory processes at a lower degree than that observed in untreated TPs. LTNPs may have mitochondrial-related alterations in adipose tissue which are greater than that observed in TPs.

Key Words: HIV, long-term nonprogressors, adipose tissue, inflammation, mitochondria

(*J Acquir Immune Defic Syndr* 2012;61:131–137)

INTRODUCTION

Current knowledge suggests that the appearance of the lipodystrophy syndrome in HIV-1–infected patients treated with antiretroviral drugs cannot be explained solely in terms of the effects of the drugs alone and that events related to the HIV-1 infection itself may also be involved.[1] This has been highlighted by the observation that some HIV-1–infected patients who are naive to antiretroviral drugs may develop adipose tissue disturbances reminiscent of lipodystrophy,[2,3] a finding that led investigators to hypothesize that HIV-1 itself could damage adipose tissue. Further experimental evidence supported this. Adipose tissue mtDNA depletion,[4] impairment of the expression of some mitochondrial genes and of the key master adipogenesis regulator peroxisome proliferator–activated receptor-gamma (PPARγ), and increased expression of some inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) and β2-microglobulin have been shown to be present in untreated HIV-1–infected patients, even without clinical fat redistribution.[5,6] Data elsewhere also suggest that HIV-1 proteins such

Received for publication January 26, 2012; accepted April 13, 2012.

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Supported by Instituto de salud Carlos III grants PI07/0976, PI08/1715, and PI10/2635, PI11/0376 and INT11/240. Fondos para el Desarrollo Europeo Regional; Programa de suport als grups de recerca AGAUR (2009) grants SGR 284, 959, and 1061; Ministerio de Ciencia e Innovación grant SAF2008-02278, SAF2012-35198; Ministerio de Sanidad, Política Social e Igualdad grant EC11-293; Red de Investigación en Sida grants RIS, RD06/006/0022 and RD06/0006/1004, ISCIII, Spain.

The authors have no conflicts of interest to disclose.

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as Vpr and Tat are responsible for promoting apoptosis and inhibiting adipogenesis.[7–12] It has consistently been demonstrated then that HIV-1, through its own products and/or secondary inflammatory phenomena, can damage adipose tissue, and that this damage may have clinical consequences. In this respect, we recently performed a study that involved untreated HIV-1–infected patients, and treated patients with lipodystrophy. Subcutaneous adipose tissue (SAT) studies indicated that disturbances in adipose tissue gene expression were present in untreated infected patients, and that these disturbances worsened with the use of antiretroviral drugs, ultimately leading to lipodystrophy.[5] The subset of untreated HIV-1–infected patients was made up of typical progressors (TPs) (that is to say, individuals with high plasma viral loads and low $CD4^+$ T-cell counts). In the present article, we discuss the results of a study performed in HIV-1–infected long-term nonprogressors (LTNPs). LTNPs are a subset of patients characterized by a long-standing HIV-1 infection together with a low viral mass, in the absence of any antiretroviral drug use.[13] They are regarded as being a model of natural self-control of HIV-1 infection, in which both host and viral factors are involved.[14]

It can be hypothesized that, because of their lower viral burden, untreated HIV-1–infected LTNPs may have a less disturbed adipogenesis, inflammation, and mitochondrial toxicity parameters in SAT than untreated HIV-1–infected TPs. However, longer exposure to infection, even at a low-grade level, may enhance deleterious processes in adipose tissue in LTNPs. To determine whether this is so, we compared the patterns of gene expression alterations in SAT from HIV-1–infected TPs and LTNPs.

METHODS

Individuals

We performed a cross-sectional case-control study which included 3 patient categories: uninfected controls (UCs), untreated HIV-1–infected LTNPs, and untreated HIV-1–infected TPs. Criteria for LTNPs were asymptomatic HIV-1 infection with a known duration of more than 15 years, a stable $CD4^+$ T-cell count persistently more than 500 cells/μL, and plasma HIV-1 viral load repeatedly less than 5000 copies/mL, in the absence of any antiretroviral treatment.[13] Untreated TPs were patients whose HIV-1 infection had progressed (ie, if they had an HIV-1 viral load more than 35,000 copies/mL, and a progressively declining $CD4^+$ T-cell count that had gone less than 350 cells/μL at least twice during the first 8 years of infection) and who had not previously received or were not currently receiving antiretroviral drugs, were free of symptoms, and had not suffered opportunistic infections before recruitment.

Uninfected controls were individuals who had elective abdominal surgery for nonmalignant conditions (usually elective cholecystectomy) and were otherwise healthy. We carefully checked that neither HIV-1–infected patients nor UCs had any conditions known to damage mitochondria, such as smoking, alcohol abuse, or liver disease, and also that they were not taking drugs known to cause mitochondrial derangement.[15] Informed consent was obtained from each participant. The project was approved by the local ethical research committees.

Methods

Plasma HIV-1 RNA concentrations were measured with the Cobas Amplicor HIV-1 Monitor Test v 1.5 using the COBAS AMPLICOR system (Roche Diagnostics, Basel, Switzerland). Blood $CD4^+$ T-cell count was assessed in a flow cytometer FACScan (Becton Dickinson Immunocytometry System, San Jose, CA), and the data acquired were analyzed using the Multiset program.

Subcutaneous fat tissue biopsy was performed in all of the subjects. An 8-mm^3 sample of abdominal subcutaneous fat was obtained by biopsy. Tissue samples were frozen in liquid nitrogen and kept at −80°C until processing. After homogenization in RLT buffer (Qiagen, Hilden, Germany), an aliquot was used to isolate DNA, with a standard phenol/chloroform extraction methodology. RNA extraction was performed using a column-affinity–based methodology (RNeasy; Qiagen), which included on-column DNA digestion (RNase-Free DNase set, Qiagen). One milligram of RNA was transcribed into cDNA using MultiScribe reverse transcriptase and random-hexamer primers (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, CA). For quantitative mRNA expression analysis, TaqMan RT-PCR was performed on the ABI PRISM 7700HT sequence detection system (Applied Biosystems). The TaqMan RT-PCR reaction was performed in a final volume of 25 μL using TaqMan Universal PCR Master Mix, No AmpErase UNG reagent, and the specific gene expression primer pair probes (Applied Biosystems).

The parameters assessed and their biological significance are detailed in Table 1. The Assay-on-Demand probes (TaqMan Gene Expression Assays; Applied Biosystems) used were 18S rRNA, Hs99999901; cytochrome c oxidase subunit IV (COIV), COX4I1, Hs00266371; nuclear respiratory factor 1, Hs00192316; PPARγ, Hs00234592; PPARγ coactivator 1α, Hs00173304; lipoprotein lipase (LPL), Hs00173425; fatty acid–binding protein 4 (FABP4), Hs00609791, TNF-α, Hs00174128; monocyte chemoattractant protein 1 (MCP-1), Hs00234140; interleukin 18 (IL-18), Hs99999040; complement component 3 (C3), Hs00163811; CD1A, Hs00381753; β2-microglobulin, Hs99999907. The primers and probes for detecting COII and mtDNA abundance assessment were designed using the Assay-by-Design system (Custom TaqMan Gene Expression Assays; Applied Biosystems), and the sequence were AAACCACTTTCACCGCTACAC (forward) and GGACGATGGGCATGAAACTGT (reverse). The FAM-labeled probe was AAATCTGTGGAGCAAACC. mtDNA was quantified using these last probe and referred to nuclear DNA as determined by the amplification of the intronless gene C/EBPalpha (Hs00269972), as previously reported.[16] Appropriate controls with no RNA, primers, or reverse transcriptase were included in each set of experiments. Each sample was run in duplicate, and the mean value of the duplicate was used to calculate the mRNA expression of the genes of interest which were normalized to that of the reference control (18S rRNA) using the comparative (2-DeltaCT) method and following the instructions of the manufacturer. Parallel calculations using the cyclophilin-A (peptidyl propyliso reverse A [PPIA]) reference gene (Hs99999904), another housekeeping gene, were performed and results were essentially the same.

TABLE 1. Explanation and Significance of the SAT mRNA Parameters Assessed

Parameter Assessed	Significance
Adipogenic/lipid	
PPARγ	Peroxisome proliferator–activated receptor-gamma. Key master regulator of adipocyte differentiation
FABP4	Fatty acid–binding protein 4. Transports fat into the adipocyte. It is a PPARγ target
LPL	Lipoprotein lipase. Captures fatty acids by adipose tissue. It is a PPARγ target
Inflammatory	
TNF-α	Tumor necrosis factor-alpha. Proinflammatory cytokine
MCP-1	Monocyte chemoattractant protein 1. Proinflammatory cytokine
IL-18	Interleukin 18. Proinflammatory cytokine
CD1A	Dendritic cell marker
β2-microglobulin	Target for TNFα and other proinflammatory cytokines
C3	C3 component of the complement system
Mitochondrial	
mtDNA	Mitochondrial DNA content
COII	Subunit II of the cytochrome oxidase. Member of the mitochondrial respiratory chain encoded by the mitochondrial DNA
COIV	Subunit IV of the cytochrome oxidase. Member of the mitochondrial respiratory chain encoded by the nuclear DNA
NRF1	Nuclear respiratory factor 1. Mitochondrial biogenesis regulator
PGC-1α	Peroxisome proliferator–activated receptor (PPAR) gamma coactivator 1α. Transcriptional coactivator involved in mitochondrial biogenesis

Statistical Analysis

Data of continuous variables of all categories were expressed as median and 25th and 75th percentiles. The Kruskal–Wallis test was performed to compare the continuous variables of the 3 categories: UC, LTNP, and TP, and the Mann–Whitney *U* test was performed to compare the results between 2 groups. The χ^2 test, with Yates if necessary, was performed to compare discrete variables. In all cases, a *P* value of less than 0.05 was considered to be statistically significant.

RESULTS

Characteristics of the Participants

Table 2 shows the main characteristics of the UCs and the 2 categories of HIV-1–infected patients studied. Groups were comparable for age and gender. As expected, the duration of HIV-1 infection, the plasma viral load, and $CD4^+$ T-cell count were significantly different between HIV-1–infected LTNPs and TPs (Table 2). As shown, our untreated LTNPs were individuals with extreme long-standing infection (more than 15 years) with preserved immune parameters and low viral burden.

Adipogenic/Lipid Parameters

Data are shown in Figure 1. Overall, PPARγ, FABP4, and LPL mRNA expression were significantly decreased in both HIV-1–infected subsets with respect to UCs. Comparison of HIV-1–infected LTNPs and HIV-1–infected TPs indicated that the latter had a significantly decreased mRNA expression of PPARγ ($P = 0.006$). LPL and FABP4 expression were nonsignificantly different between these 2 subsets.

Inflammatory Parameters

In abdominal wall SAT, TNF-α, IL-18, β2-MCG, MCP-1, CD1A, and C3 mRNA were assessed (Fig. 2). The behavior of the various parameters assessed was somewhat different. TNF-α, IL-18, and β2-MCG showed the following trend: both HIV-1–infected subsets had significantly greater mRNA expression than UCs. In HIV-1–infected subjects, IL-18 was significantly greater in TPs than in LTNPs, and TNF-α and β2-MCG were nonsignificantly different in these 2 subsets. C3 was significantly increased in UCs with respect to both HIV-1–infected subsets. Otherwise, CD1A and MCP-1 showed a similar trend to C3, but the differences were nonsignificant (Fig. 2).

Mitochondrial Parameters

We assessed mtDNA content and COII and COIV mRNA levels. The last 2 are mitochondrial respiratory chain proteins encoded by mitochondrial and nuclear DNA, respectively. COII and COIV were significantly lower in LTNPs than in UCs and TPs. TPs had lower COII and COIV mRNA expression than UCs, but the difference was significant only for COIV. This may reflect the effect of HIV-1 on diffuse cell damage. The levels of mtDNA in adipose tissue were significantly lower in LTNPs respect to UCs, but not in TPs (Fig. 3). In fact, mtDNA levels in LTNPs were significantly lower than in TPs. There were no significant differences between the 3 subsets of individuals for the transcript levels of the mitochondrial biogenesis regulator nuclear respiratory factor 1. The mRNA expression for the PPARγ coactivator 1α, coactivator of mitochondrial biogenesis, was significantly increased only in TP.

DISCUSSION

Previous studies have demonstrated that HIV-1 infection itself produces mitochondrial disturbances in the peripheral blood mononuclear cells of untreated infected patients.[17] It has also been reported that disturbance is lower in LTNP than in TP.[18] In the present study, we demonstrate for the first time that this also occurs in adipose tissue, since untreated HIV-1–infected LTNP may have limited but significant derangement in some adipogenic/lipid, inflammatory, and mitochondrial parameters in SAT. This derangement is lower than that observed in untreated TPs, with the exception of mitochondrial damage. Hence, our data suggest that HIV-1 itself may damage adipose tissue and that the derangement correlate, at least partially, with both the viral burden and the duration of infection. Whether the derangement of

TABLE 2. Demographic and HIV-1–Related Data of the Individuals Assessed. Data are Expressed as Median (25th–75th)

Variable	UCs (n = 10)	Untreated HIV-1–Infected Long-Term Nonprogressors (n = 10)	Untreated HIV-1–Infected Typical Progressors (n = 10)	*P*
Age, yrs (±SD)	37 (32.8–40.5)	42 (38–43)	36 (29–41.5)	0.09
Sex (male, %)	80	50	80	0.24
Time since HIV-1 infection diagnosis (yrs)	—	16 (15.6–17.6)	4 (3–8.9)	<0.001
HIV-1 RNA (log_{10}, copies per milliliter)	—	1.7 (1.3–3.3)	4.8 (4.6–5.3)	<0.001
$CD4^+$ T-cell count (cells per cubic millimeter)	—	658 (581–782)	296 (180–328)	<0.001

adipogenic, inflammatory, and mitochondrial parameters in adipose tissue starts shortly after infection or develops over time remains unknown.

Our observations should be borne in mind when studying the pathogenesis of lipodystrophy, the exact mechanisms of which still remain obscure. At one time, lipodystrophy was thought to be an adverse effect of protease inhibitors,[19] because of the close relationship between the widespread use of this drug family and the recognition of the syndrome. To date, the reason why some protease inhibitors produce lipodystrophy (and particularly the lipohypertrophic component of the syndrome) has not been satisfactorily explained, but perturbations in the process of adipogenesis have been proposed.[20] It has also been shown that some nucleoside reverse transcriptase inhibitors are also associated with lipodystrophy (and especially with lipoatrophy).[21] The association is particularly strong with thymidine analogues—zidovudine and, especially, stavudine[22]—which is thought to be because of the mitochondriotoxic potential of these drugs, which favors adipocyte apoptosis.[23] Thus, the role of antiretroviral drugs in lipodystrophy has consistently been demonstrated, although there is no consensus on which molecular mechanisms are involved. Otherwise, currently available data suggest that HIV-1 itself could also be involved in adipose tissue derangements. In this respect, basic science studies have shown that some proteins produced by the virus (Vpr, Tat, Nef, among others) may have deleterious effects on the adipose tissue.[7,8,10,24–26] Moreover, HIV-1 transgene expression in mice causes changes in adipose tissue that are reminiscent of those in patients with lipodystrophy, particularly early pretreatment changes.[27] Clinical association studies confirmed that HIV-1–infected subjects, in the absence of any antiretroviral treatment, show marked derangements in adipose tissue, which include an increased local inflammatory milieu and perturbations in adipogenesis and in mitochondrial parameters.[5] Before the present study, it was not known whether the pattern and intensity of the derangement produced by HIV-1 in adipose tissue varies in different clinical patterns of the infection, but we have provided here evidence that suggests that even a low-invasive pattern of infection (represented by the untreated LTNPs) damages adipose tissue, although to a lesser extent than untreated TPs. A consistently low viral burden over a long period of time, as is the case of LTNPs, produces significant adipose tissue derangement.

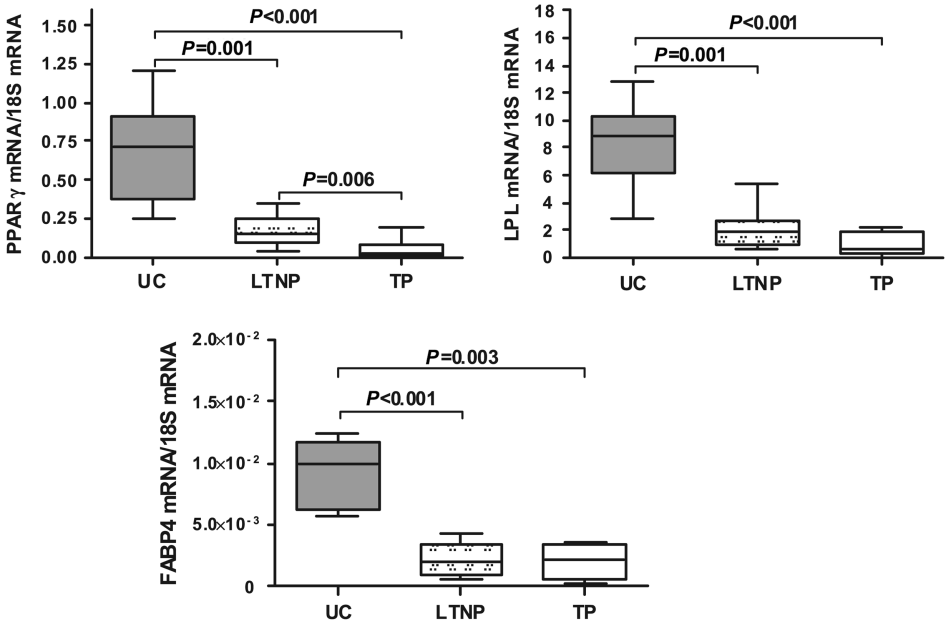

FIGURE 1. Adipocyte differentiation and lipid metabolism marker gene expression in SAT from UCs, untreated HIV-1–infected LTNPs, and untreated HIV-1–infected typical progressors (TPs). The figure shows the box-and-whisker plot representing specific mRNA concentrations. The line within the box marks the median, the upper boundary of the box indicates the 75th percentile, and the lower boundary the 25th percentile. Error bars above and below the box indicate the 100th and 0th percentiles. For comparison, the *P* values are indicated above the boxes when statistical significance is $P < 0.05$.

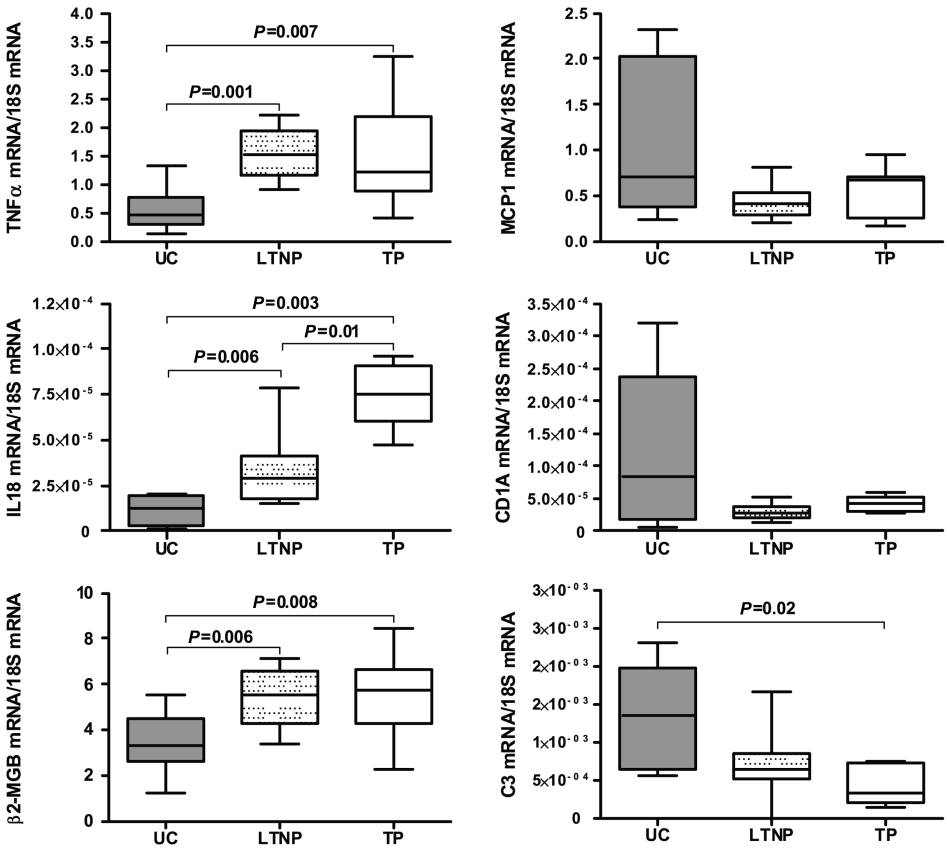

FIGURE 2. Inflammation-related marker gene expression in SAT from UCs, untreated HIV-1–infected LTNPs, and untreated HIV-1–infected TPs. The figure shows the box-and-whisker plot representing specific mRNA concentration. The line within the box marks the median, the upper boundary of the box indicates the 75th percentile, and the lower boundary the 25th percentile. Error bars above and below the box indicate the 100th and 0th percentiles. For comparison, the *P* values are indicated above the boxes when statistical significance is $P < 0.05$.

A detailed analysis of our observations suggests that HIV-1 itself markedly impairs the expression of proteins involved in adipocyte differentiation, such as PPARγ, LPL, and FABP4. The duration of the infection and the amount of viral mass do not seem to affect LPL and FABP4, whereas a long-standing infection reduces PPARγ expression. This suggests that this key master regulator of the adipocyte differentiation process is more sensitive to the duration of the infection than to the viral mass.

As far as inflammatory parameters are concerned, we[28,29] and others[30,31] have observed that the inflammatory milieu is increased in the subcutaneous fat of treated infected patients with lipodystrophy. Giralt et al[5] reported that HIV-1 infection itself produces some degree of inflammation in adipose tissue. We have shown here that this already exists in LTNPs, although to a lesser extent, particularly with respect to IL-18. Our findings suggest that even a low-invasive HIV-1 infection, exemplified by LTNPs, produces some degree of inflammatory derengement in the host. Because inflammation has been correlated with lipodystrophy[32] and both premature atherosclerosis[33] and aging,[34] our data seem to suggest that clinical studies should consider the possibility of prescribing antiretroviral treatment earlier than that is currently recommended in guidelines.

As far as the disturbances in the adipose tissue mitochondrial parameters assessed in our study are concerned, the data confirm that untreated HIV-1–infected patients show mild mitochondrial damage in adipose tissue.[5,35] We have also observed that long-standing low-invasive HIV-1 infection (the LTNP model), alters mitochondrial-related parameters more markedly than a shorter infection with higher viral mass (the TP model). This suggests that mitochondrial damage is related more to the duration of the infection than to the amount of the infection (viral mass), as occurs with other causes of mitochondrial derangement. In fact, distinct pathologies caused by mitochondrial alterations (ie, mtDNA genetic diseases) are usually progressive degenerative diseases, with a marked worsening of symptoms over time.[36] We should point out that in our groups of patients, despite a trend to lower mtDNA levels in adipose tissue from TP, only LTNP showed a significant depletion of mtDNA with respect to UC. There have been reports of unaltered mtDNA levels[37,38] or mtDNA depletion[35] in HIV-1–infected TP. Maybe these varied findings could be explained by slight differences in the duration of HIV-1 infection in the untreated patients assessed.

We acknowledge that our study has some limitations. First, the cross-sectional nature of our design provides associations, not causality. Second, there are some inconsistencies in our results because as we have discussed above, some parameters were damaged, others were not, and others were by means no clear or difficult to interpret because of considerable variation in some subsets. Of note, the data for C3, CD1A, and MCP-1 in UC varied considerably, and this may render comparisons somewhat difficult to interpret. Third, the low number of patients assessed suggests prudence when interpreting our data. Some nonsignificant comparisons (because of underpower) might become significant if replicated in further

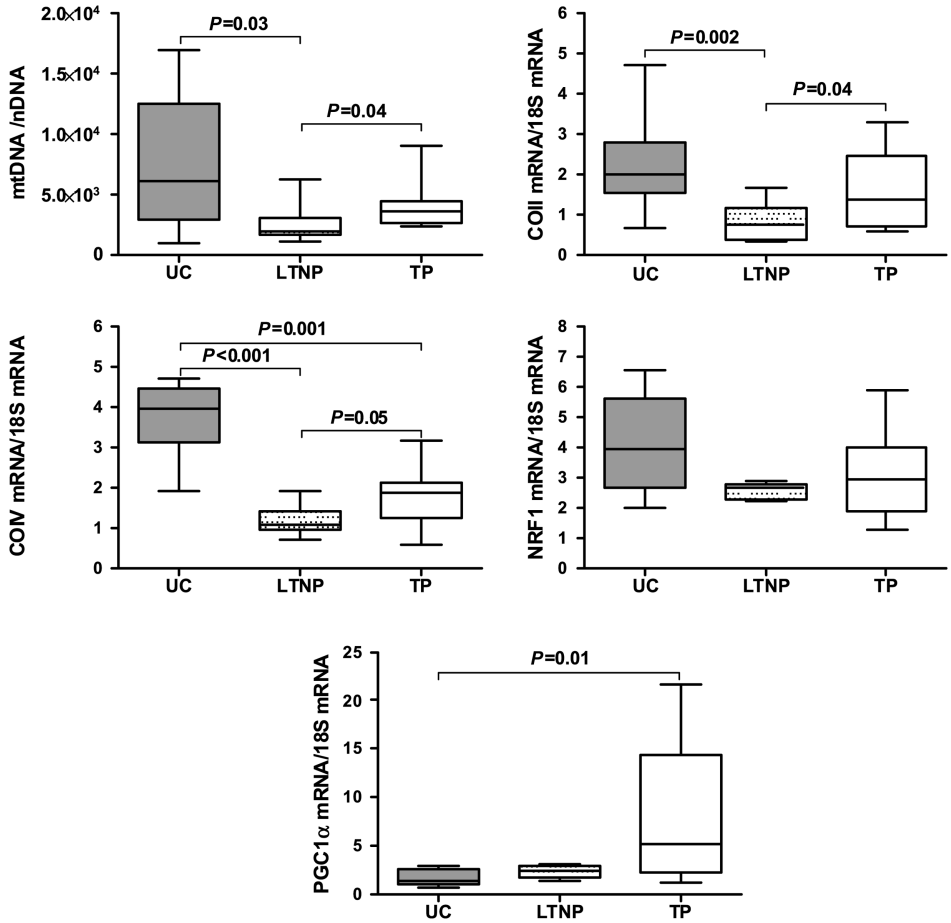

FIGURE 3. Mitochondrial function marker gene expression and mitochondrial DNA levels in SAT from UCs, untreated HIV-1–infected LTNPs, and untreated HIV-1–infected TPs. The figure shows the box-and-whisker plot representing specific mRNA or mtDNA concentration. The line within the box marks the median, the upper boundary of the box indicates the 75th percentile, and the lower boundary the 25th percentile. Error bars above and below the box indicate the 100th and 0th percentiles. For comparison, the *P* values are indicated above the boxes when statistical significance is $P < 0.05$.

larger studies. It should be highlighted, however, that it is not easy to perform a study such as this because it is difficult to convince individuals who are completely asymptomatic and not taking antiretroviral drugs to permit a SAT biopsy.

In summary, the present study suggests that HIV-1 by itself may damage the expression of diverse adipogenic/lipid, inflammatory, and mitochondrial parameters in adipose tissue, and that this is even true in patients with an extremely low-invasive pattern of infection, such as LTNP. These patients, although they have low-grade viremia, may have significant mRNA gene adipose-tissue disturbances.

ACKNOWLEDGMENTS

We would like to thank our patients who kindly provided samples of SAT. John Bates improved the English text. The comments and criticisms performed by the anonymous reviewers helped us to improve the manuscript.

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Lipotoxicity on the Basis of Metabolic Syndrome and Lipodystrophy in HIV-1-Infected Patients Under Antiretroviral Treatment

Revista: Current Pharmaceutical Design. 2010 Oct; 16(30):3371-8 (REVIEW)

PMID: 20687888

Título: La lipotoxicidad como base del síndrome metabólico y la lipodistrofia en pacientes infectados por HIV-1 bajo terapia HAART

El desarrollo de fármacos antirretrovirales eficaces que minimicen los efectos adversos es un reto de la terapia contra el virus HIV-1. Las alteraciones metabólicas reminiscentes del síndrome metabólico y la lipodistrofia aparecen a menudo en los pacientes HIV-1 tratados con terapia HAART. La etiopatogénesis de estas alteraciones es compleja, pero recientemente la lipotoxicidad ha emergido como un factor clave para poder explicar el síndrome metabólico de estos pacientes, de manera similar a lo que se ha observado en enfermedades como la obesidad y las lipodistrofias genéticas. Los medicamentos antirretrovirales de las diferentes familias pueden provocar este fenómeno de lipotoxicidad aumentando la lipólisis, reprimiendo la adipogénesis y aumentando la apoptosis de los adipocitos, lo cual lleva a reducir la capacidad del tejido adiposo subcutáneo de aumentar para almacenar las reservas de grasa. De este modo los ácidos grasos no pueden ser almacenados correctamente como triglicéridos en el tejido adiposo subcutáneo y se acumulan en el tejido adiposo visceral y los órganos y tejidos, como el páncreas, músculo o hígado, originando un patrón de alteraciones metabólicas asociadas, principalmente la resistencia a insulina, por la acumulación ectópica de grasa. La respuesta inflamatoria evocada por los efectos combinados de los medicamentos antirretrovirales y la infección por el virus HIV-1, también contribuyen a la lipotoxicidad, ya que la acción de las citoquinas pro-inflamatorias aumentan la actividad lipolítica en el tejido adiposo e impiden la adipogénesis. Minimizar el efecto lipolítico de los medicamentos de la terapia HAART es esencial para reducir las alteraciones metabólicas de los pacientes tratados. Las estrategias farmacológicas que reduzcan la lipotoxicidad y promueven la expansibilidad del tejido adiposo, serían adecuadas para evitar alteraciones metabólicas en los pacientes infectados por HIV-1 en tratamiento HAART.

Lipotoxicity on the Basis of Metabolic Syndrome and Lipodystrophy in HIV-1-Infected Patients Under Antiretroviral Treatment

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Abstract: The development of efficacious antiretroviral drugs that minimize adverse effects is a current challenge in HIV-1 therapy. Metabolic alterations reminiscent of the metabolic syndrome and overt lipodystrophy appear often in HIV-1-infected patients undergoing antiretroviral treatment. The etiopathogenesis of these alterations is complex, but lipotoxicity has recently emerged as a key concept for explaining the metabolic syndrome in HIV-1-infected patients, similarly to what has been observed in diseases such as obesity and genetic lipodystrophies. Antiretroviral drugs from distinct drug families may directly elicit such lipotoxic phenomena, *via* increased lipolysis, enhanced adipocyte apoptosis and impaired adipogenesis, which collectively lead to a reduced capacity of subcutaneous adipose tissue to enlarge to meet fat storage requirements. Thus, fatty acids that cannot be properly stored as triglycerides in subcutaneous adipose tissue are expected to accumulate in visceral fat as well as in organs and tissues, such as the pancreas, muscle and liver, leading to the pattern of metabolic alterations associated with abnormal ectopic fat accumulation, mainly insulin resistance. Inflammatory responses, evoked by the combined effects of antiretroviral drugs and the underlying HIV-1 infection, also contribute to lipotoxicity, reflecting the action of pro-inflammatory cytokines that enhance lipolytic activity in adipose tissue and impair adipogenesis. Minimizing the lipotoxic action of antiretroviral drugs is ultimately essential in reducing metabolic alterations in treated patients. Moreover, pharmacological strategies that reduce lipotoxicity and promote adipose tissue expandability can be expected to ameliorate the overall metabolic abnormalities in HIV-1-infected, antiretroviral-treated patients.

Keywords: Lipotoxicity, lipodystrophy, HIV, fatty acids, adipose tissue.

1. INTRODUCTION: IS LIPOTOXICITY A PIVOTAL CONTRIBUTOR TO THE METABOLIC SYNDROME IN HIV-1-INFECTED PATIENTS UNDERGOING HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART)?

Lipotoxicity is increasingly highlighted as a possible explanation for the development of metabolic syndrome and diabetes in multiple pathological situations, from obesity to lipodystrophies of distinct origin. According to the lipotoxicity theory, excess availability of fatty acids or a limited capacity to metabolize them in organs and tissues elicits most of the alterations that are characteristic of the metabolic syndrome, specially insulin resistance. The toxicity of fatty acids toward β-cells may lead to altered insulin production; increased availability and, ultimately, increased accumulation of fat into skeletal muscle tends to promote insulin resistance; and fatty acid levels in liver that exceed the capacity of this organ to oxidize or export them lead to the increased accumulation of fat that is often associated with the metabolic syndrome. All of these events could result from alterations in fat metabolism or excess intake of fat-enriched nutrients, but they could also arise as a result of intrinsic alterations in the capacity of adipose tissue to store fat and thereby buffer against excess accumulation of fatty acids in other tissues and organs [1].

A remarkable number of HIV-1 infected patients undergoing antiretroviral treatment develop signs of metabolic syndrome, typified by insulin resistance and hyperlipidemia. This phenomenon often occurs in association with lipoatrophy of peripheral, subcutaneous, adipose tissue, but not of visceral adipose tissue, which is often enlarged. The ultimate pathogenic mechanisms leading to HAART-associated lipodystrophy syndrome (HALS) in HIV-1-infected patients undergoing HAART are not fully understood [2,3]. One hypothesis consistent with the appearance of the metabolic syndrome in HALS patients is lipotoxicity resulting from limitations in the capacity of subcutaneous fat to store the appropriate amounts of fat and the subsequent diversion of fatty acids to ectopic sites. In the present article, we review the available data on lipotoxicity as a mechanism for explaining the metabolic syndrome that appears in association with antiretroviral treatment of HIV-1-infected patients. Identification of lipotoxicity as an essential ethiopathogenic event in metabolic alterations in HIV-1-infected patients may help orient pharmacological and nutritional strategies designed to ameliorate the metabolic status of these patients.

2. LIPOTOXICITY AS A PATHOGENIC MECHANISM FOR METABOLIC SYNDROME IN OBESITY AND GENETIC LIPODYSTROPHY

Several arguments in support of lipotoxicity as a major contributor to the metabolic syndrome in distinct human pathologies come from the paradoxically common metabolic alterations found in obesity and in lipodystrophies of genetic origin. According to the lipotoxicity theory, the fat stored in adipose tissue is biologically inert and the observed metabolic alterations are primarily caused by the increased exposure of cells to non-esterified fatty acids. Thus, in obese patients, it is not the amount of stored fat in adipose tissue that elicits metabolic dysfunctions, but rather the balance between the availability of these fatty acids to tissues and organs and the capacity of these organs to eliminate them through triglyceride storage in adipose tissue, or oxidation [4]. A complementary concept is the idea that there is a threshold for adipose tissue expandability; once reached, as in obese individuals, appropriate storage of fatty acids in their inert, esterified form inside adipocytes is impaired. Evidence in support of the lipodystrophy hypothesis is provided by the existence of a small subset of obese patients who remain metabolically "normal". These patients exhibit massive obesity without signs of metabolic syndrome [5,6], and it is claimed that these individuals exhibit a strong adipose tissue expandability, a capacity that allows them to cope with excess fatty acids even

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1381-6128/10 $55.00+.00

when the adipose tissue mass is already very large. These studies in obese patients have lead to the concept that individual differences in the expandability of adipose tissue and threshold of maximal fat storage in adipose tissue may control the flow of fatty acids to other organs and thereby determine the extent of lipotoxicity.

Another line of evidence in support of the concept that lipotoxicity underlies metabolic syndrome is the presence of profound metabolic alterations in patients with genetically-determined lipodystrophy, which paradoxically exhibits several features similar to those that occur in obesity. Genetic forms of lipodystrophy are rare (although likely underdiagnosed), and are characterized by a generalized or partial deficiency of adipose tissue [7]. Unlike the reduction in fat mass in leanness, which reflects diminished lipid accumulation in adipose tissues, the decrease in adipose tissue in lipodystrophy is characterized by dysfunctional or absent mature adipocytes.

Congenital generalized lipodystrophy, or Berardinelli-Seip syndrome (BSCL), is an autosomal-recessive disease characterized by a near-complete absence of adipose tissue from birth that results primarily from mutations in either the gene encoding 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2), an enzyme involved in the biosynthesis of triglycerides and phospholipids [8], or of the gene encoding seipin (BSCL2), an endoplasmic reticulum protein involved in lipid droplet biology [9]. Caveolin-1, a main component of plasma membrane lipid rafts [10] has recently been identified as a third BSCL gene. BSCL patients develop metabolic complications that include severe hypertriglyceridemia, insulin resistance, diabetes, hepatic steatosis and markedly diminished serum levels of leptin and adiponectin [11].

Several types of familial partial lipodystrophy, mainly related to mutations in the genes encoding nuclear lamin A/C (LMNA/LMNC) and peroxisome proliferator activated receptor-γ (PPARγ), have also been reported. These partial lipodystrophies are more common and appear later (around puberty) than congenital generalized lipodystrophy. Adipose tissue pathology in familial partial lipodystrophy is related to decreased adipose tissue in some areas and/or altered fat distribution, with predominant loss of limb fat depots but generally preserved truncal fat [12]. In these patients, the loss of adipose tissue results in an abnormal capacity to properly store lipids and secrete adipokines, which may contribute to the pathogenesis of metabolic diseases.

Thus, either a total or partial lack of adipose tissue storage capacity causes an increase in circulating lipid levels and is associated with ectopic lipid accumulation in non-fat tissues such as liver, skeletal muscle and pancreas. These alterations lead to non-alcoholic fatty liver disease, hepatic and muscle insulin resistance and impaired pancreatic islet function, and thereby contribute to the development of type-2 diabetes. Adipokine deficiency further contributes to metabolic alterations, (e.g. hyperphagia) owing to an associated leptin deficiency, which also contributes to positive energy balance and potential lipotoxicity through increased fat intake [13]. Studies in patients with lipodystrophy of genetic origin suggest that the loss of adipose tissue may be directly related to the appearance of metabolic alterations reminiscent of those that occur in HALS patients.

3. MOUSE MODELS OF GENETIC LIPODYSTROPHY CONFIRM A MAJOR ROLE FOR ADIPOSE TISSUE EXPANDABILITY IN THE DEVELOPMENT OF METABOLIC SYNDROME

The conclusions reached in studies on human lipodystrophies of genetic origin have been largely confirmed in rodent models of lipodystrophy [14]. Several different knock-out mice designed to mimic human generalized or partial genetic lipodystrophies have been generated. Like their human counter-parts, AGAPT2-null mice display generalized loss of adipose tissue, fatty liver, extreme insulin resistance and diabetes [15]. In contrast, none of the knock-out mouse models for partial genetic lipodystrophy mimic human disorders. LMNA-null mice manifest muscular dystrophy but not lipodystrophy [16], and PPARγ-null mice die embryonically (see below). However, transgenic expression of a human mutant form of LMNA in mouse adipose tissue results in age-dependent partial lipodystrophy that is reminiscent of human type 2 familial partial lipodystrophy (FPLD2) [17]. At the molecular level, this has been proposed to reflect reduced adipocyte differentiation capacity of LMNA-mutant adipose tissue. Although some discrepancies in gender and fat distribution are seen between human FPLD2 and this murine model, metabolic consequences such as hepatic steatosis and insulin resistance are similarly manifested [17].

Many other mouse models of lipodystrophy in which different genes involved in adipocyte differentiation are specifically targeted have been developed [for reviews, see 14,18-20]. Targeted disruption of transcription factor CCAAT/enhancer binding protein alpha (C/EBPα) profoundly alters adipose tissue differentiation [21,22]. These mice die shortly after birth due to hepatic dysfunction [21]. Transgenic expression of C/EBPα specifically in liver rescues neonatal lethality [23]. These mice lack all white adipose depots, but not brown adipose depots, indicating that although C/EBPα expression is an absolute requirement for white adipose tissue, its absence can be compensated for in brown fat development, probably by C/EBPβ [22]. This lipodystrophic mouse model also exhibits hyperinsulinemia, hyperlipemia and fatty liver [23].

Numerous mouse models with defective or altered levels of PPARγ have contributed to our understanding of the key role of this transcription factor in adipocyte differentiation and lipid metabolism [24]. Genetically engineered mice with global PPARγ deficiency in adulthood exhibit severe lipoatrophy and insulin resistance [25]. Heterozygous PPARγ-null mice and hypomorphic PPARγ mice with reduced expression of PPARγ also exhibit significantly reduced body fat, plasma leptin and adiponectin levels and insulin resistance [26,27]. Several transgenic mice bearing human mutant PPARγ forms found in FPLD patients have been developed. Although these mice exhibit hypertension and reduced fat mass, extreme insulin resistance only appears when crossed with leptin-deficient ob/ob mice [28]. In summary, genetic modification of key components of the adipocyte differentiation machinery has given rise to mice with a limited (or absent) ability to develop adipose cells, and therefore with an intrinsic impairment in the capacity to store fat in adipose tissue.

Other lipodystrophic murine models have been created through targeted disruption of adipocyte differentiation or viability. Some of these models are conditional, such as the aP2-DTA mice, in which lipoatrophy was engineered by inducing adipocyte death through adipocyte-specific expression of diphtheria toxin [29], and FAT-ATTAC mice, which undergo transient lipoatrophy after temporally controlled induction of adipocyte apoptosis [30]. These mice lose adipose tissue mass in adulthood and thereafter develop dyslipemia, insulin resistance and hepatic steatosis. The lipodystrophy of FAT-ATTAC mice can be reversed by recovery of adipocyte function, as evidenced by an improved adipokine profiles and alleviation of metabolic disturbances [30]. One of the first models to be developed was the generalized lipodystrophic A-ZIP/F mouse, in which a dominant-negative transcription factor that interferes with adipogenic C/EBPs was transgenically expressed in adipose tissue [31]. These transgenic mice lack white adipose tissue and have reduced brown fat; as consequence, they manifest many of the metabolic complications seen in human congenital generalized lipodystrophy. Transplantation of white fat pads into these mice improves hepatic steatosis and insulin sensitivity thereby demonstrating that these alterations are at least partially due to the lack of adipose tissue [32].

Another early onset model of generalized lipodystrophy is a transgenic mouse in which expression of a constitutively active form of sterol response element binding protein 1c (aP2-SREBP1c-

mice) is driven specifically in adipose tissue [33]. SREBP1c is a transcription factor involved in the regulation of lipogenesis that must be enzymatically processed to be active. Unexpectedly, aP2-SREBP1c-mice almost completely lack white adipose tissue and develop hyperlipemia, fatty liver and insulin resistance. Because leptin levels are extremely low in these lipodystrophic models (adipose tissue secretes leptin in proportion to its size), early studies tested the metabolic effects of infusing leptin. In the first such study, infusion of leptin to aP2-SREBP1c-mice resulting in dramatic metabolic improvements that were not limited to reduction of hyperphagia [34]. Leptin administration was also shown to provide metabolic benefits in A-ZIP/F mice [35]. These studies prompted to therapeutic trials of leptin replacement, an approach that is now used in the treatment of human genetic-lipodystrophies [36] (see below).

Finally, it must be kept in mind that several mouse models show a lean, but not a lipodystrophic, phenotype that is a consequence of an increase in energy expenditure rather than an adipocyte dysfunction. These models exhibit reduced adiposity but are protected from insulin resistance and hepatic steatosis because their reduced, but 'still expandable', adipose tissue together with efficient fatty acid oxidation prevents ectopic lipid accumulation and interference with insulin action [18,19].

In conclusion, these murine models support the notion that the capacity of adipose tissue to store fat appropriately is essential to prevent ectopic fat storage and tissue damage associated with excess fat accumulation in sites other than adipose cells. Although mouse models do not necessarily mimic the genetic basis of human lipodystrophies or their specific pattern of fat loss, they display almost all the metabolic disturbances found in human lipodystrophy, and thus constitute excellent research tools for studying novel therapeutic approaches.

4. ANTIRETROVIRAL DRUGS AND HIV-1 INFECTION AS PRIMARY AGENTS RESPONSIBLE FOR LIPOTOXICITY IN HALS PATIENTS.

There are several indications that lipotoxicity may be a key contributor to the metabolic alterations that occur in HIV-1-infected patients, especially in association with lipodystrophy. In patients with peripheral lipoatrophy, ectopic fat deposition is commonly reported. HALS patients often show hepatic steatosis, in association with insulin resistance. Abnormal accumulation of fat also takes place in skeletal muscle in association with impaired glucose disposal in HALS patients.

Antiretroviral drugs are likely to be the primary agents responsible for promoting alterations that ultimately lead to lipotoxicity. However, ascertaining the relative contribution of individual drugs from the distinct antiretroviral drug families is a complex undertaking. Among nucleoside-analog reverse transcriptase inhibitors (NRTIs), thymidine-analog drugs in particular are known to be particularly related to peripheral lipoatrophy and the associated metabolic alterations [37]. This appears to be due to the mitochondrial toxicity of this type of drug [38], which may contribute to impaired local oxidation of fat and diversion of fatty acids to the circulation. Moreover, there are several reports supporting a specific action of this type of drugs in promoting lipolysis and the subsequent efflux of non-esterified fatty acids from adipose tissue [39,40]. *In vitro* studies have reported that NRTIs, such as stavudine and zidovudine, impair adipocyte differentiation [41], although to a lesser extent than protease inhibitors (PIs) (see below). The most common non-nucleoside reverse transcriptase inhibitors (NNRTIs), efavirenz and nevirapine, have not been traditionally linked to the appearance of lipodystrophy or of profound metabolic alterations. However, recent clinical trials have established that efavirenz favors fat loss when included as a component of antiretroviral therapy cocktails [42,43]. In contrast, several reports have indicated that shifting PI-based or NRTI-based regimes to one containing nevirapine modestly improves the lipid profile of adult [44] and pediatric [45] patients. The beneficial effects of nevirapine on the lipid profile of treated patients [46, 47], is associated with an increase in HDL-cholesterol and the total cholesterol/HDL-cholesterol ratio. In addition, efavirenz profoundly impairs adipogenic differentiation of rodent and human cells "*in vitro*" whereas nevirapine does not [48]. Finally, PIs have been generally thought to induce multiple metabolic alterations reminiscent of the metabolic syndrome, particularly insulin resistance, although these drugs do not appear to be particularly associated with lipoatrophy [49]. *In vitro* studies have shown that most PIs impair adipogenic differentiation, and promote lipolysis in adipocytes [50].The impairment of adipogenic differentiation common to both PIs and NRTIs may be a major mechanistic contributor to the lipotoxic action of these antiretroviral drugs, as this action could limit the capacity of adipose tissues of patients to expand appropriately to cope with excess fatty acids.

It should be noted that PIs have been reported to cause endoplasmic reticulum (ER) stress [49,51-53] characterized by altered ER protein-folding functionality in response to multiple cellular stressors, including non-esterified fatty acids. ER stress evokes a coordinated intracellular response that results in attenuation of protein synthesis and degradation of misfolded proteins. Several reports have indicated that fatty acids exert lipotoxicity in non-adipose tissue cells by activating this process; for instance, fatty acids are known to promote ER stress in pancreatic β-cells, leading to decreased synthesis of insulin. Activation of this process by PIs may make an additive contribution to the action of fatty acids, resulting in enhanced activation of this lipotoxicity-related intracellular process.

A much smaller number of studies have sought to determine whether the underlying HIV-1 infection, -the other primary etiopathogenic agent responsible for metabolic alterations in HIV-1-infected, antiretroviral-treated patients- is involved in lipotoxicity. Before the HAART era, several studies found altered lipid metabolism in HIV-1-infected patients, even in the absence of AIDS or wasting syndrome [54]. It has also been reported that pre-HAART ("naïve") HIV-1-infected patients show signs of reduced body weight and dyslipemia that cannot be attributed to malnutrition or opportunistic illnesses. Especially relevant in the context of lipotoxicity are the observations that untreated HIV-1-infected patients exhibit enhanced lipolysis prior to the onset of illness and, after HAART, HIV-1 infection-induced changes in lipolysis remain and are distinct from subsequent HAART-related lipodystrophy [55].

Several transgenic rodents models in which HIV-1-derived proteins are expressed have shown altered lipid metabolism and signs of impaired adipose tissue development. *In vitro* studies have also shown that HIV-1- derived proteins such as Vpr and Nef-1 may alter adipocyte differentiation by interfering with master transcription factors of adipogenesis, such as PPARγ [56]. Thus, processes directly related to HIV-1 infection may contribute to lipotoxicity along with impaired differentiation of adipocytes and limited adipose expandability. The extent to which HALS in patients undergoing HAART is due to drug treatment alone or to interactions of drug treatments with characteristic features of the HIV-1-infected organism is not yet clear. Assessments of non-infected volunteers exposed to antiretroviral drugs have shown several alterations in metabolism that are reminiscent of those observed in patients, but ethical considerations limit the duration of such studies, making it difficult to reach definitive conclusions [57].

5. SECONDARY AGENTS INVOLVED IN LIPOTOXICITY IN HALS PATIENTS: INFLAMMATION, IMPAIRED ADIPOGENESIS, MITOCHONDRIAL TOXICITY AND ENHANCEMENT OF FATTY ACID FLOW

The combined action of drugs and the underlying HIV-1 infection in patients elicits multiple cellular and metabolic alterations

that, in turn, produces secondary events that promote lipotoxicity. Complex cross-talk among multiple pathogenic events converges to evoke the lipotoxic scenario characterized by impaired adipose tissue expandability and enhanced fatty acid flux from adipose tissue to other tissues and organs.

A reduction in the levels of PPARγ has been a common finding in subcutaneous adipose tissue biopsies from HALS patients [58,59]. This observation, which reflects both the action of some HIV-1 proteins on PPARγ expression, as noted above, and the effects of certain antiretroviral drugs impairing PPARγ expression, is consistent with defects in adipose depot expandability and therefore capacity to store fatty acids, at least in the subcutaneous depot of adipose tissue. A second effect that favors the impaired expandability of adipose tissue is the pro-inflammatory environment in adipose tissue of HALS patients. The enhanced production of multiple pro-inflammatory cytokines, induced in adipose tissue depots by primary agents that cause HALS, contributes to the down-regulation of master adipogenesis genes such as PPARγ [3] and therefore further limits the capacity of the adipose depot to replenish itself.

Enhanced metabolic flow of non-esterified fatty acids, a key element in the lipotoxicity theory, has been reported in HALS patients. Specifically, high rates of lipolysis have been observed in HIV-1 patients under distinct drug treatment regimens [39,40]. Treatment with acipimox, an inhibitor of lipolysis, has been reported to improve insulin sensitivity and dyslipidemia in HIV-1 patients [60-62] and to attenuate atherogenesis in rodent models of antiretroviral drug-induced dyslipidemia [63]. The action of multiple primary and secondary agents -the lipolytic effect of HIV-1 infection, irrespective of HAART; the lipolytic action of antiretroviral drug, noted above; and the lipolytic action of pro-inflammatory cytokines- are likely to converge to cause the increase lipolysis observed in HALS patients.

One final factor that contributes to peripheral lipid deposition is the relative uptake capacity among tissues reflecting their respective lipoprotein lipase (LPL) activities. Accordingly, elevated LPL activity in adipose tissue would protect against lipotoxicity at the expense of increased adipose tissue expansion. In contrast, active LPL in tissues such as skeletal muscle would result in increased lipid deposition and, consequently, lipotoxicity, if the lipid oxidative capacity of the tissue was not concomitantly improved. In this context, LPL expression is reported to be decreased in biopsies of subcutaneous adipose tissue from HALS patients [59]. Moreover, PI treatment has been associated with reduced LPL-mediated lipolysis of lipoprotein triacylglycerols and therefore, an impaired fatty acid uptake in adipose tissue [64]. The fact that the LPL gene is a target of PPARγ [65] must also be taken into account; thus, the impaired expression of PPARγ expression in adipose tissue from HALS patients may contribute to the reduced fatty acid uptake capacity in adipose tissue through its effects on LPL.

Finally, the well known mitochondrial toxicity caused by antiretroviral drugs, mainly NRTIs, may contribute to lipotoxicity [38]. A mechanism to impair excess fatty acid in non-adipose tissues is just promoting their oxidation. The fact that antiretroviral treatment tends to cause a reduction in mitochondrial oxidative functions is expected to contribute to excess free fatty acid availability. On the other hand, several reports indicate an abnormal induction of the expression of the uncoupling protein-1 gene caused by antiretroviral drugs *in vitro* [66] as well as in HALS-associated lipomatosis [67]. Uncoupling protein-1 is a marker of brown fat, a particular type of adipose tissue that burns fatty acids instead of storing them. However, there is no evidence that such induction results in functional brown adipose tissue capable of high rates of fatty acid oxidation in HALS patients.

6. EXPANDABILITY OF ADIPOSE TISSUE: A POTENTIAL TARGET FOR AMELIORATING METABOLIC DISRUPTIONS IN HALS PATIENTS?

The fact that the lipotoxic effects associated with the limited capacity of adipose tissue to store fatty acids may be a main ethiopathogenic factor in metabolic alterations of HALS patients suggests that therapies designed to maximize adipose tissue expandability may be a potential strategy for normalizing the metabolic profile of these patients.

At first glance, thiazolidinediones may appear to be ideal for this purpose. Because they act as insulin sensitizers, the thiazolidinediones rosiglitazone and pioglitazone are currently used to treat type-2 diabetes. Moreover, thiazolidinediones activate PPARγ, the master transcriptional regulator of adipogenesis, thereby promoting the differentiation of novel adipocytes from precursor cells and expanding the capacity of adipose depots to store esterified fatty acids [68]. In fact, it has been hypothesized that the improvement in insulin sensitivity and other metabolic parameters produced by thiazolidinediones is due, in whole or in part, to this adipose tissue expansion-promoting effect and associated reduction in lipotoxicity. However, thiazolidinediones have shown limited effectiveness in the treatment of lipodystrophy and the associated metabolic syndrome in HIV-1-infected patients. Most reports indicate a substantial improvement in insulin sensitivity, as in non-HIV-1-infected diabetic patients, but lipoatrophy as such is little improved [69,70]. It is likely that the low levels of PPARγ, the mediator or thiazolidinedione action, in adipose tissue from HALS patients makes the tissue poorly sensitive to thiazolidinedione treatment.

Another possible treatment option is leptin. Leptin, which is primarily produced by adipose tissue, creates satiety signals in the hypothalamus and promotes catabolic pathways in adipose tissue and other tissues and organs [1]. In several rodent models of lipodystrophy that exhibit abnormally low leptin levels, leptin replacement has been shown to result in dramatic metabolic improvement [34]. Moreover, leptin replacement has proven to be highly effective in the treatment of patients with congenital generalized lipodystrophy, largely through a reduction in food intake but also through an increase in the oxidation of lipids ectopically deposited in skeletal muscle and liver [71,72]. In HALS patients leptin levels are reported to be unaltered or abnormally reduced [73]. Results from the few pilot studies that have tested leptin treatment in HALS patients have shown an improvement in dyslipidemia and in insulin resistance as well as a reduction in lipolysis in agreement with a reduction in lipotoxicity [74].

Finally, another approach to treat HALS and the associated metabolic alterations has been the use of growth hormone (GH) or agents eliciting GH release. GH replacement was early envisaged to the light of some of the alterations in HALS patients are associated with GH axis deficiency [75]. Treatment of patients with GH has been reported to improve slighly peripheral lipoatrophy and to a higher extent visceral adiposity. However, these effects were dependent on the GH dose use, and low GH doses minimizing side effects were also less effective in controlling adipose alterations. [76,77]. An important finding in the context of lipotoxicity is that a major effect of GH treatment of HALS patients is a reduction in lipolysis rate [78]. More recently, analogues of GH-releasing-hormone (GHRH) have been assayed, such as “tesamorelin”, and some improvement in fat distribution has been reported with a lesser impairment in glycemic control [79].

7. CONCLUSIONS

In summary, currently available pharmacological strategies for minimizing lipotoxic action of antiretroviral drugs are quite limited; thus, identifying novel targets and developing new drugs capable of

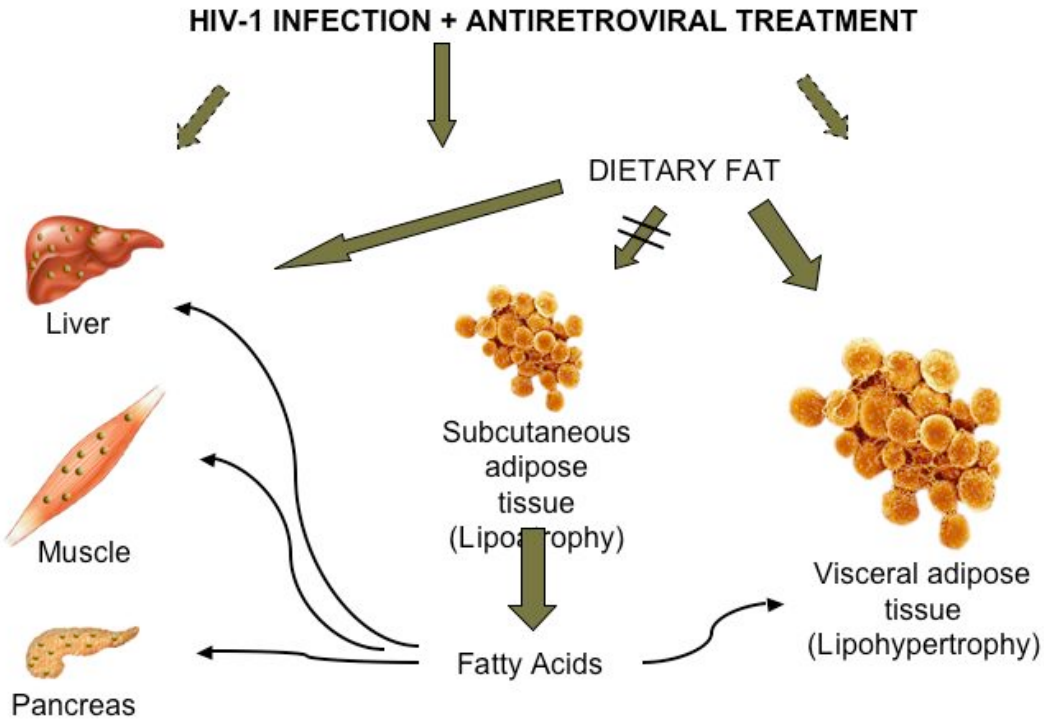

Fig (1). Schematic representation of the events associated with lipotoxicity in HALS. HIV-1 infection-related events (inflammation, direct action of HIV-1-encoded proteins) as well as antiretroviral drugs promote lipoatrophy in subcutaneous adipose tissue, thus leading to the active release of lipids as well as to the impaired capacity to store dietary lipids. Released fatty acids and dietary fat accumulate in visceral adipose tissue, thus leading to visceral lipohypertrophy and the metabolic derangements associated with visceral obesity. Moreover, fatty acids from lipoatrophic adipose tissue as well as lipids from other tissues and dietary intake accumulate in non-adipose tissues (e.g., liver, muscle, pancreas) and exert lipotoxic effects, in addition to the direct deleterious effects on these tissues caused by HIV-1 infection and drug treatment.

modulating lipotoxicity may be essential to reduce metabolic alterations in treated patients. Similarly to other pathologic conditions in which lipotoxicity is involved (e.g., obesity and genetic lipodystrophies), novel pharmacological solutions can be expected to involve distinct strategies to: a) modulate adipose tissue plasticity and thereby improve the capacity of pre-adipocytes to differentiate and increase the lipid-buffering capacity; b) favor endogenous oxidation of fatty acids in adipose tissue depots over their release into the circulation, a strategy referred to as promoting "brown-versus-white" characteristics in adipose depots; c) favor repartitioning of fat away from the tissues and organs that are more susceptible to lipotoxic effects; and d) promote fat oxidation in non-adipose tissues and organs, and thereby avoid ectopic fat accumulation. All these strategies should also be a part of efforts to minimize the adverse metabolic effects of novel antiretroviral drugs.

ABBREVIATIONS

AGPAT2	=	1-acylglycerol-3-phosphate O-acyltransferase 2
BSCL	=	Berardinelli-Seip syndrome
C/EBP	=	CCAAT/enhancer binding protein
ER	=	Endoplasmic reticulum
FPLD2	=	Type 2 familial partial lipodystrophy
GH	=	Growth hormone
GHRH	=	GH-releasing hormone
HAART	=	Highly active antiretroviral treatment
HALS	=	HAART-associated lipodystrophy syndrome
LMNA/LMNC	=	Lamin A/C
LPL	=	Lipoprotein lipase
NRTI	=	Nucleoside-analog reverse transcriptase inhibitor
NNRTI	=	Non-nucleoside-analog reverse transcriptase inhibitor
PPARγ	=	Peroxisome proliferator activated receptor-γ
PI	=	Protease inhibitor
SREBP1c	=	Sterol response element binding protein 1c

ACKNOWLEDGEMENTS

Supported by grants from Ministerio de Ciencia e Innovación (SAF2008- 01896), Instituto de Salud Carlos III (PI08/1715) and Red de Investigación en SIDA (RIS RD06/006). Thanks are given to V.Carreño for support in the design of the Fig. (**1**).

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Received: July 7, 2010 Accepted: July 14, 2010

Nadir CD4 T Cell Count as Predictor and High CD4 T Cell Intrinsic Apoptosis as Final Mechanism of Poor CD4 T Cell Recovery in Virologically Suppressed HIV-Infected Patients: Clinical Implications

Revista: Clinical Infectious Diseases. 2010 May 1;50(9):1300-8.

PMID: 20367229

Título: Contaje nadir de células CD4 T como predictor y alto ratio de apoptosis intrínseco de las células CD4 T como mecanismo final, para la pobre recuperación de los niveles de CD4 T en pacientes infectados por HIV-1 con supresión virológica: Implicaciones clínicas

A pesar de que la terapia HAART mejora la respuesta inmune, algunos pacientes infectados por el HIV-1 bajo tratamiento presentan una recuperación de células CD4 T insatisfactoria a pesar de lograr el descenso de la carga viral, aumentando la mortalidad y la morbilidad. Estos pacientes presentaron unos mayores niveles de marcadores de activación, principalmente en células CD4 T y mayores ratios de muerte celular espontanea. Los ratios de activación y ratios de muerte de células CD4 T fueron los mejores factores predictores de la recuperación inmune junto con el contaje nadir de células CD4 T. Los pacientes que eran tratados con inhibidores de proteasas eran más afines a mostrarse discordantes y mostraban unas ratios de activación y de muerte celular de células CD4 T, así como un contaje nadir de células CD4 T más bajo. DE todas maneras, el análisis multivariable no detectó ningún efecto de los inhibidores de proteasas sobre la recuperación inmune. No se observaron diferencias entre el uso de “Truvada” y el uso de “Kivexa”. La apoptosis de células CD4 T por vías intrínsecas representa el mecanismo de la recuperación inmune no satisfactoria y debería ser diana para mejorar la terapia de los pacientes discordantes. El valor predictivo del contaje nadir de las células CD4 T para una pobre recuperación inmune nos lleva a considerar la opción de comenzar la terapia antes. No se observaron diferencias entre los diferentes fármacos en cuanto a respuesta inmune.

Nadir CD4 T Cell Count as Predictor and High CD4 T Cell Intrinsic Apoptosis as Final Mechanism of Poor CD4 T Cell Recovery in Virologically Suppressed HIV-Infected Patients: Clinical Implications

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Background. Although antiretroviral therapy improves immune response, some human immunodeficiency virus–infected patients present unsatisfactory CD4 T cell recovery despite achieving viral suppression, resulting in increased morbidity and mortality.

Methods. Cross-sectional, case-control study to characterize the mechanism and to identify predictive factors of poor immune response. We included 230 patients who were receiving highly active antiretroviral therapy and who had a viral load <50 copies/mL for >2 years; 95 were "discordant" (case patients; CD4 T cell count always <350 cells/μL), and 135 were "concordant" (control subjects). Activation markers, CD4 T cell death (necrosis, intrinsic apoptosis, and extrinsic apoptosis), and caspase-3 were measured. Clinical parameters, particularly antiretroviral combinations, were correlated with immune recovery.

Results. Discordant patients showed higher levels of activation markers, mainly in CD4 T cells ($P < .001$), and higher rates of spontaneous cell death ($P < .001$). Rates of activation and rates of CD4 T cell death (mainly by intrinsic apoptosis) were the best predictive factors for immune recovery, along with nadir CD4 T cell count. Patients who were receiving a protease inhibitor–based regimen were more likely to be discordant and showed higher rates of activation ($P = .011$), higher rates of CD4 T cell death ($P = .033$), and a lower nadir CD4 T cell count ($P < .001$). Multivariate analysis, however, ruled out any effect of protease inhibitors on immune recovery. No differences were observed between the use of tenofovir-emtricitabine (Truvada) and the use of abacavir-lamivudine (Kivexa).

Conclusions. CD4 T cell apoptosis by the intrinsic pathway represents the determinant mechanism of the unsatisfactory immune recovery and should be targeted to manage therapy for discordant patients. The predictive value of low nadir CD4 T cell count for a poor immune recovery led us to consider starting antiretroviral therapy earlier. No differences were observed among antiretrovirals in terms of immune recovery.

Clinicians who care for individuals infected with human immunodeficiency virus (HIV) are concerned about the lack of immune recovery in some virologically suppressed patients. This group, called discordant or immunological nonresponder patients, is defined as patients who are receiving highly active antiretroviral therapy (HAART) and who maintain suppression of HIV replication without an adequate CD4 T cell count recovery. Unlike full responders, discordant patients are at increased risk of clinical progression to AIDS-related and non–AIDS-related illnesses and death [1–7]. Cohort studies reveal a substantial prevalence of immunological nonresponders among patients who are receiving HAART, ranging from 17% to 40%, depending on the study criteria and the population [5, 8, 9]. These data indicate that a high number of treated HIV-infected patients are at risk of clinical progression.

Received 24 November 2009; accepted 20 January 2010; electronically published 30 March 2010.

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Clinical Infectious Diseases 2010; 50(9):1300–1308

1058-4838/2010/5009-0016$15.00
DOI: 10.1086/651689

From a clinical point of view, older age [10, 11], lower nadir CD4 T cell count [1, 10, 12], and hepatitis C virus (HCV) coinfection [13–15] are some of the most relevant predictive factors for a discordant response. Critical questions, such as the choice of the most appropriate antiretroviral therapy in these cases, remain unanswered.

There has been a great deal of effort aimed at understanding the virological and immunological basis for poor immune recovery. In general, low production of new T cells, due to impaired bone marrow or thymus function [1, 16–18], and increased CD4 T cell destruction, revealed by the high sensitivity to cell death ex vivo [17, 18], have been shown to contribute to this failure. Also, the unbalanced T cell homeostasis may be influenced by the existence of latent, undetectable, ongoing viral replication [19], microbial translocation from the gastrointestinal lumen [20], and adverse effects of antiretroviral drugs at the mitochondrial level [21]. Each of these conditions can influence the production, activation, and death of CD4 T cells and thus determine the discordant response.

In this regard, we have recently observed that an increased rate of CD4 T cell death appears to be a determining factor for poor immune recovery in a group of 95 immune-discordant patients, compared with 135 responders (Massanella et al, unpublished data). Such increased destruction of CD4 T cells is closely related to immune hyperactivation, because activated cells are prone to apoptosis ex vivo [22]. However, the ultimate cause of the unfavorable immune response has not been identified. A better knowledge of the definitive pathogenic mechanism and the factors influencing CD4 T cell recovery would help investigators to design adequate strategies focused on improving the immunological response in discordant patients and to determine the optimal therapeutic approach for them.

The present study was designed on the basis of the hypotheses that CD4 T cell apoptosis determines the immune recovery after HAART and that some clinical conditions, such as coinfection or a specific antiretroviral treatment, are triggering factors for discordant response. Accordingly, the aims of this study were to identify the CD4 T cell death pathways that limit recovery of CD4 T cells and to determine their association with clinical parameters and antiretroviral regimens.

MATERIALS AND METHODS

Study design and population. We performed a cross-sectional, case-control study to analyze the role of cell death in discordant immune response. A 12-month period of inclusion (1 November 2007–31 October 2008) was chosen, with the assumption that during this period all discordant patients in our cohort could be identified. We screened 247 consecutive patients who attended our HIV Outpatient Unit, and 230 met the following inclusion criteria: (1) confirmed diagnosis of HIV infection, (2) continual HAART for >2 years that included 2 nucleoside reverse-transcriptase inhibitors (NRTIs) plus 1 ritonavir-boosted protease inhibitor (PI) or 1 nonnucleoside reverse-transcriptase inhibitor (NNRTI) (nevirapine or efavirenz), and (3) sustained undetectable levels of HIV-1 RNA (plasma viral load, <50 copies/mL) for >2 years (minimum number of determinations, 4). The exclusion criteria were chemotherapy, treatment with interferon and/or ribavirin, a history of opportunistic infection during the previous 2 years, and the presence of decompensated liver cirrhosis.

We considered to be discordant (ie, having favorable virological but unfavorable immunological response) those patients with a CD4 T cell count that was always <350 cells/μL. Conversely, concordant patients (favorable virological and immunological response) showed a current CD4 T cell count >400 cells/μL. These criteria ensured that patients with a CD4 T cell count from 350 to 400 cells/μL were excluded from the study, to clearly distinguish the 2 immune scenarios (discordant and concordant response).

The Institutional Review Board of our center approved the study (EO code: EO-07–024). All patients provided their written informed consent.

Study objectives and end points. The main objective of the study was to characterize the cell death pathways that lead to a poor CD4 T cell recovery. Our main end points were the rates of total cell death, necrosis, and apoptosis (intrinsic and extrinsic), compared between discordant and concordant patients. Another objective was the comparison of the levels of CD4 T cell activation and of caspase-3 between discordant and concordant patients. Finally, we investigated the influence of clinical parameters on immune recovery, particularly the effect of different antiretroviral combinations, according to whether they included a PI, an NNRTI, or the most frequently used coformulated NRTIs tenofovir-emtricitabine (Truvada) or abacavir-lamivudine (Kivexa).

Assessments. Information on patient characteristics and HIV-related data was collected from medical records. The status of infection with hepatitis B virus (HBV) or with HCV was also retrieved from this patient history database.

A single blood sample was drawn from each participant. Blood was immediately stained and processed. Plasma was obtained by centrifugation of blood at 1200 *g* for 10 minutes and was stored at −80°C. Peripheral blood mononuclear cells (PBMCs) were obtained from cell concentrates layered on Ficoll–Hypaque density gradients (Atom Reactiva) and were used immediately for ex vivo cell death assays or were frozen for caspase-3 determinations.

Cell death was evaluated by culturing aliquots of 200,000 PBMCs in 96-well plates in 100 μL of Roswell Park Memorial Institute medium (containing 10% fetal calf serum) for 0, 1, and 4 days in the absence or presence of the pancaspase inhibitor Z-VAD-fmk (R & D Systems). Cells were analyzed in

an LSRII flow cytometer (Becton Dickinson) after incubation with 40 nM of the potentiometric mitochondrial probe $DiOC_6$ (Invitrogen), 5 μg/mL propidium iodide (Sigma), and CD3-APC-Cy7, CD4-APC, and CD8-PE-Cy7 antibodies. Total cell death was calculated as the percentage of cells that showed low $DiOC_6$ staining in control cultures [23].

On day 1, we performed additional analyses of necrotic cell death (caspase-independent), which was defined as the percentage of propidium iodide–stained cells in cultures that contained Z-VAD-fmk. Apoptotic cell death was defined as caspase-dependent death and was calculated by subtracting necrosis from total cell death. Intrinsic apoptosis was defined as the percentage of cells that showed low $DiOC_6$ staining and that remained negative for propidium iodide in the presence of Z-VAD-fmk. Extrinsic apoptosis was calculated as the difference between total and intrinsic apoptosis. Caspase-3 activity was determined using 15 μg of total protein from PBMC lysates with a fluorometric assay (Ac-DEVD-AMC, CASPASE-3; Becton Dickinson).

CD4 and CD8 T cell immunophenotypes were assessed by staining fresh blood samples with the following antibody combination: CD95-FITC, PD-1-PE, HLA-DR-PerCP, CD3-APC-Cy7, CD4-APC, and CD8-PE-Cy7, which was designed to evaluate activation. A control staining and a control combination that contained CD3-APC-Cy7, CD4-APC, and CD8-PE-Cy7 antibodies were performed for all samples. Cells were acquired in the LSRII flow cytometer and were analyzed with FlowJo software (Tree Star).

Soluble CD14 levels, which are a surrogate marker for bacterial translocation [24], were quantified in all plasma samples by means of commercially available enzyme-linked immunosorbent assays (Diaclone). Plasma samples were diluted (1:50) and were tested in duplicate.

Statistical analysis. Continuous variables were expressed as the median (interquartile range [IQR]) and were compared using nonparametric tests (Mann-Whitney for nonpaired data and Wilcoxon for paired data), because the parameters were not normally distributed. Discrete variables were described as number of patients (percentage), and the χ^2 or Fisher exact test was used, as appropriate. The Pearson correlation coefficient was calculated to assess the association between the apoptosis parameters and the clinical variables.

Univariate and multivariate logistic regressions were fitted to predict the probability of discordance by considering the following as explanatory variables: age, sex, route of transmission, time with HIV infection, time receiving antiretroviral therapy, antiretroviral used at baseline, time with suppressed viral load, nadir CD4 T cell count, baseline CD4 T cell count, coinfection with HBV or HCV, and hepatitis C viral load. The models were also fitted after adjustment for use of PI and for HCV coinfection.

Statistical analyses were performed using SPSS, version 15.0 (SPSS), with univariate 2-tailed significance levels of .05. Graphs were plotted with GraphPad Prism, version 5 (GraphPad).

RESULTS

Patient characteristics. A total of 230 patients were included in the study: 95 were defined as discordant and 135 as concordant. Most demographic and clinical parameters were well balanced among both groups (Table 1). However, minimal but significant differences were observed in the time with viral load <50 copies/mL and in CD8 T cell absolute counts. As expected, significantly lower CD4 T cell counts (absolute and percentage), significantly lower nadir CD4 T cell counts, and a significantly higher number of patients with nadir CD4 T cell counts <200 cells/μL were observed in the discordant group ($P < .001$ for all). Patients with HCV coinfection and patients who were receiving PI-containing regimens were more likely to be found in the discordant group (Table 1).

Analysis of activation and destruction and characterization of cell death pathways. Cell death was measured in CD4 and CD8 T cells immediately after cell purification (day 0) and after 1 or 4 days of ex vivo culture. In particular, levels of an activation marker ($CD4^+HLA\text{-}DR^+CD95^+$) and rates of total death in CD4 T cells were significantly higher in discordant patients ($P < .001$) (Table 1 and Figure 1A). Conversely, although CD8 T cells showed increased levels of activation markers ($CD8^+HLA\text{-}DR^+CD95^+$) (Table 1), no significant differences in rates of ex vivo cell death were observed in this subset (Figure 1A).

With regard to the mechanisms of cell death, the discordant group showed higher rates of necrosis and of total apoptosis (intrinsic and extrinsic) in CD4 T cells at day 1, compared with the corresponding rates for concordant patients (Figure 1B). In contrast, CD8 T cells from discordant patients showed higher rates of necrosis and intrinsic apoptosis but lower rates of extrinsic apoptosis (Figure 1C). Levels of caspase-3 were significantly higher in discordant patients (10,409 absorbance units/mg protein [IQR, 7018–16,203 absorbance units/mg protein]) than in concordant patients (9035 absorbance units/mg protein [IQR, 5080–13,358 absorbance units/mg protein]) ($P = .050$).

Rates of CD4 T cell intrinsic apoptosis showed a stronger correlation than did rates of necrosis or extrinsic apoptosis with the expression of different markers of activation, such as the frequency of $CD38^+CD45RA^-$ CD4 T cells (r, 0.617; $P < .001$) and $HLA\text{-}DR^+CD95^+$ CD4 T cells (r, 0.523; $P < .001$). This finding suggests that activated cells die mainly by intrinsic apoptosis.

Clinical parameters that influenced cell death and discordant response. On inclusion, 55% of participants were receiving a stable PI-based regimen, mainly atazanavir (44.3%) and lopinavir-ritonavir (44.3%), and 45% were receiving an NNRTI-based regimen. Patients who were receiving a PI-con-

Table 1. HIV-Related Characteristics and Activation Markers for Concordant and Discordant Patients

Variable	Concordant patients (n = 135)	Discordant patients (n = 95)	P
Age, years	43 (39–50)	46 (42–50)	.061
Male sex, no. (%) of patients	100 (74)	78 (82)	.200
Time since HIV diagnosis, months	155 (108–197)	170 (88–242)	.310
Time receiving ART, months	131 (95–161)	114.5 (55–169)	.165
Time with VL <50 copies/mL, months	49 (31–73)	40 (27–60)	.030
Nadir CD4 T cell count, cells/μL	234 (132–319)	71 (28–135)	<.001
Nadir CD4 T cell count <200 cells/μL, no. (%) of patients	61 (45)	85 (90)	<.001
Absolute CD4 T cell count, cells/μL	632 (480–796)	249 (200–319)	<.001
CD4 T cell count, %	30 (26–36)	18 (14–21)	<.001
Time to achieve current CD4 T cell count, months[a]	131 (95–157)	113 (55–169)	.200
Absolute CD8 T cell count, cells/μL	805 (648–1112)	724 (510–986)	<.001
CD8 T cell count, %	51 (45–58)	41 (35–48)	.023
Current ART, no. (%) of patients			
PI	50 (37)	53 (55)	.007
NNRTI	84 (63)	43 (45)	.015
TDF-FTC (Truvada)	68 (50)	47 (49)	.187
ABV-3TC (Kivexa)	28 (21)	23 (24)	.165
HCV coinfection, no. (%) of patients	43 (32)	44 (46)	.028
HBV coinfection, no. (%) of patients	6 (4)	4 (4)	>.99
$CD8^+HLA\text{-}DR^+CD95^+$, % of CD8 T cells	8.2 (4.3–11.6)	12.4 (6.2–19.6)	<.001
$CD4^+HLA\text{-}DR^+CD95^+$, % of CD4 T cells	4.7 (3.2–6.9)	10.1 (6.7–19.2)	<.001

NOTE. Data are median (interquartile range), unless otherwise indicated. All participants had a human immunodeficiency viral load <50 copies/mL for >2 years. Discordant patients were defined by a CD4 T cell count always <350 cells/μL. Concordant patients were defined by a CD4 T cell count always >400 cells/μL. ABV, abacavir; ART, antiretroviral treatment; FTC, emtricitabine; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; TDF, tenofovir; VL, viral load; 3TC, lamivudine.

[a] Time from starting antiretroviral to current CD4 T cell count.

taining regimen were mostly in the discordant group (Table 1) and showed a lower nadir CD4 T cell count, a higher proportion of patients with nadir CD4 T cell count <200 cells/μL, a longer period with HIV infection, a shorter time with viral suppression, and more common HCV coinfection (Table 2). Significant differences were also seen in levels of activation markers ($CD8^+CD38^+CD45RA^-$, $P = .011$), rates of CD4 T cell death ($P = .033$), levels of soluble CD14 ($P = .002$), and rates of total ($P = .041$), intrinsic ($P = .038$), and extrinsic ($P = .033$) apoptosis (Table 2). CD8 T cells showed similar characteristics in both groups (data not shown).

No differences were observed between patients who were receiving tenofovir-emtricitabine and patients who were receiving abacavir-lamivudine with regard to immune recovery, T cell production, activation, or destruction (data not shown).

Compared with patients without HCV coinfection, patients with HCV coinfection were more likely to be discordant (Table 1) and presented a lower nadir CD4 T cell count ($P = .021$), longer time with HIV infection ($P = .017$), and higher levels of immune markers ($HLA\text{-}DR^+CD95^+$ CD4 T-cells, $P = .003$; and $CD38^+CD45RA^-$ CD8 T-cells, $P = .017$) and of soluble CD14 ($P < .001$). CD4 T cell destruction and production were similar between patients with and patients without HCV coinfection.

Univariate and multivariate analyses. In the univariate analysis (Figure 2A), there were statistically significant associations between poor immune recovery and low nadir CD4 T cell count, HCV coinfection, and PI use. The rate of death in the CD8 T cell subset was unrelated to or poorly predictive of the discordant phenotype. However, the same parameters measured in CD4 T cells were statistically significant. Of note, the rate of CD4 T cell death at day 0 and the rates of necrosis and intrinsic apoptosis at day 1 were the best predictors of discordance. No association was seen with age, sex, time with HIV infection, time with viral suppression, or type of NNRTI used.

In the multivariate analysis (Figure 2B), discordance was more likely in patients who presented a low nadir CD4 T cell count (odds ratio [OR], 1.739; 95% confidence interval [CI], 1.351–2.129; $P < .001$), high rates of necrosis in CD4 T cells (OR, 1.515; 95% CI, 1.053–2.180; $P = .025$), and high rates of CD4 T cell intrinsic apoptosis at day 1 (OR, 2.266; 95% CI, 1.455–3.531; $P < .001$).

These increased risks changed slightly after adjustment for PI use: low CD4 nadir T cell counts (OR, 1.653; 95% CI, 1.351–

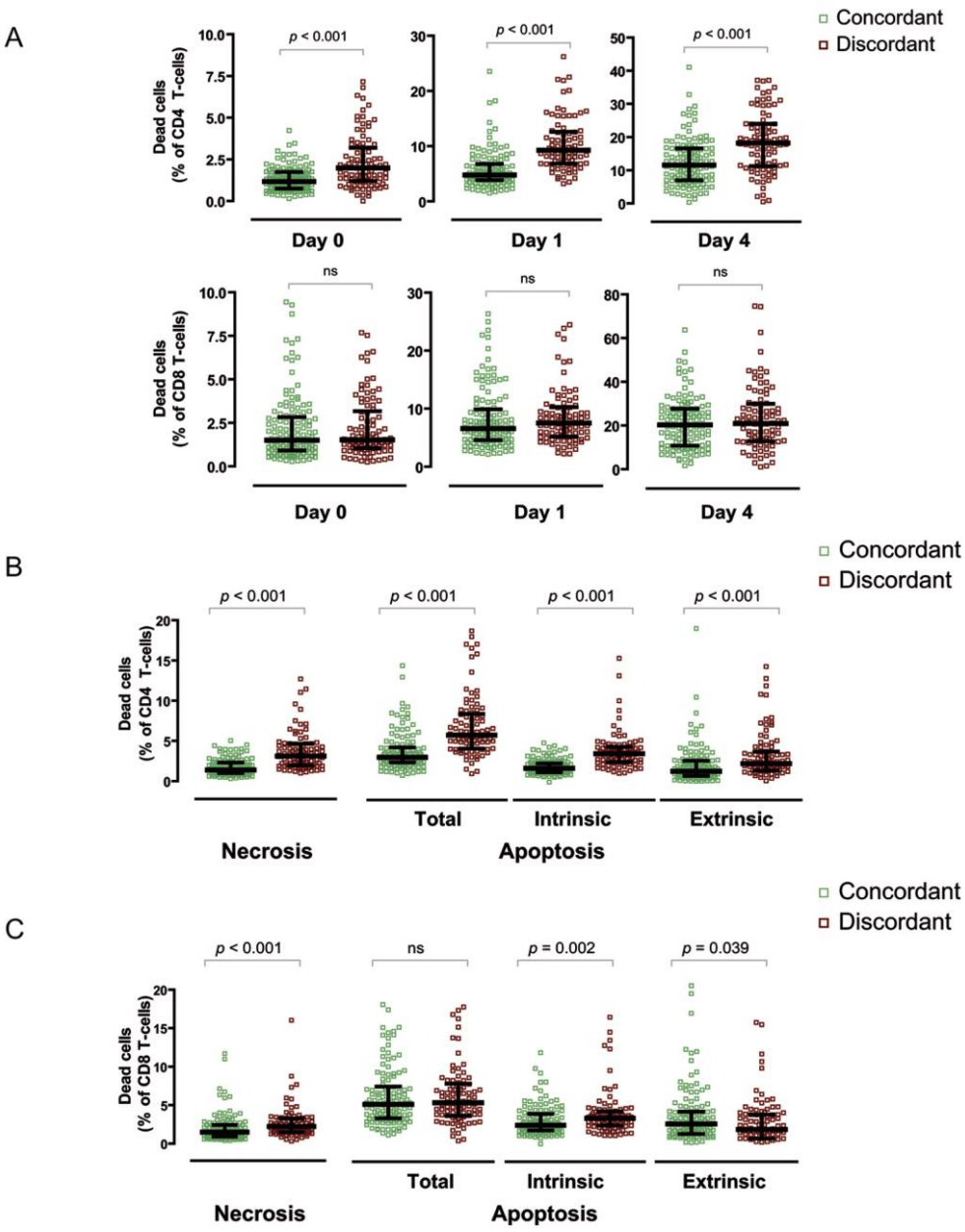

Figure 1. Analysis of cell death mechanisms. Total cell death in CD4 T cells and CD8 T cells was evaluated after 0, 1, and 4 days ex vivo cultures of peripheral blood mononuclear cells (PBMCs) *(panel A)*. Different mechanisms of cell death (necrosis, total apoptosis, intrinsic apoptosis, and extrinsic apoptosis) in CD4 T cells *(panel B)* and in CD8 T cells *(panel C)* were analyzed after 1 day ex vivo cultures of PBMCs. Individual measures of concordant patients *(green symbols)* or discordant patients *(red symbols)* with the median values *(black lines)* and interquartile ranges *(black bars)* are shown. *P* values were calculated with the Mann-Whitney test.

2.129; $P < .001$), high rates of necrosis of CD4 T cells at day 1 (OR, 1.523; 95% CI, 1.057–2.194; $P = .024$), and high rates of CD4 T cell intrinsic apoptosis (OR, 2.277; 95% CI, 1.458–3.555; $P < .001$). Because the criterion for the identification of discordant patients was based on absolute CD4 T cell counts, we investigated the role of intrinsic apoptosis in CD4 T cell recovery after stratification of all patients ($n = 230$) according to their increase in CD4 T cells (difference between their current and nadir CD4 T cell counts). Higher increases in CD4 T cells were associated with lower intrinsic apoptosis ($P < .001$) (Figure 2C).

DISCUSSION

A decreased thymic production and an increased activation and death of CD4 T cells are some of the proposed mechanisms to

Table 2. Characteristics of HIV-Infected Patients according to Regimen Based on Protease Inhibitor (PI) or Nonnucleoside Reverse-Transcriptase Inhibitor (NNRTI)

Characteristic	PI-based regimen (n = 103)	NNRTI-based regimen (n = 127)	P
Age, years	44 (41–48)	45 (40–51)	.613
Male sex, no. (%) of patients	78 (76)	100 (79)	.636
Time since HIV diagnosis, months	175 (123–230)	143 (27–206)	.017
Time with VL <50 copies/mL, months	38 (23–63)	51 (34–74)	.001
Nadir CD4 T cell count, cells/μL	92 (39–185)	189 (91–304)	<.001
Nadir CD4 T cell count <200 cells/μL, no. (%) of patients	80 (78)	66 (52)	<.001
HCV coinfection, no. (%) of patients	55 (53)	32 (25)	<.001
Discordant, no. (%) of patients	58 (56)	47 (37)	.007
$CD4^+CD45RA^+CD31^+$, % of CD4 T cells	17.4 (8.5–27.5)	15.6 (7.4–22.8)	.148
$CD4^+HLA\text{-}DR^+CD95^+$, % of CD4 T cells	7.0 (3.9–12.6)	6.1 (4.2–8.9)	.225
$CD8^+CD38^+CD45RA^-$, % of CD8 T cells	28.0 (18.7–39.3)	22.9 (16.8–31.4)	.011
Soluble CD14, μg/mL	8.9 (7.7–10.5)	8.0 (6.9–9.3)	.002
Total cell death, % of CD4 T cells	6.95 (4.74–10.33)	6.12 (4.14–8.93)	.033
Necrosis, % of CD4 T cells	2.24 (1.34–3.50)	1.89 (1.21–3.28)	.127
Apoptosis, % of CD4 T cells	3.79 (2.56–5.64)	3.32 (2.09–4.89)	.041
Intrinsic apoptosis, % of CD4 T cells	2.45 (1.72–3.76)	2.08 (1.44–3.36)	.038
Extrinsic apoptosis, % of CD4 T cells	2.08 (1.17–3.81)	1.59 (0.85–2.78)	.033

NOTE. Data are median (interquartile range), unless otherwise indicated. HCV, hepatitis C virus; HIV, human immunodeficiency virus; VL, viral load.

explain the failure to repopulate CD4 T cells in some HIV-infected patients despite an adequate virologic response to HAART [5]. Our findings support these theories and point to intrinsic apoptosis of CD4 T cell death as the predominant mechanism of cell destruction and the determining factor of discordant immune response. Clinical implications that emerge from our findings may help to answer 2 major questions about HIV infection: when to start antiretroviral therapy and what specific therapy to choose.

An increased rate of T lymphocyte cell death is one of the many adverse consequences of HIV infection and a major factor contributing to immune deterioration [25]. Cell death can be observed in multiple cell types, particularly in CD4 T cells, in which apoptosis plays a determinant role [22, 26, 27]. Antiretroviral treatment induces a decrease in rates of apoptosis as a result of a reduction in viral replication, which leads to decreased levels of viral proteins that are implicated in apoptosis and immune activation. This reduction contributes to the immune recovery that is associated with HAART. However, ~30% of patients present a discordant response to treatment, maintaining a low CD4 T cell count despite viral suppression [5, 8, 9]. This discordant immune response, accompanied by the worse outcome and the faster clinical evolution observed in these patients, has been the cause of a wide investigation [1–7].

Although the origins of the activated phenotype and of the increase in rates of CD4 T cell death remain unclear in discordant patients, there is enough evidence to confirm the strong link between activation and cell death, characterized by the high tendency of activated cells to undergo apoptosis [22]. Higher caspase-3 levels were observed in our discordant group, thus supporting higher rates of total apoptosis in these patients. In addition, our analysis shows that intrinsic apoptosis is the predominant pathway of activated human CD4 T cell destruction, as reported for animal models [28], although the extrinsic apoptotic pathway and necrosis are also involved. Interestingly, higher rates of necrosis and intrinsic apoptosis of CD8 T cells were also observed in discordant patients, which shows higher activation rates in this subset. This fact reinforces the notion that increased rates of activation are linked to higher sensitivity to intrinsic pathways of apoptosis.

In clinical terms, the knowledge of the pathogenesis of CD4 T cell destruction is a basic step in the design of successful strategies to improve immune reconstitution in immunological nonresponders. The predominant implication of intrinsic apoptosis in poor immune recovery makes sense, because antiretroviral drugs may inhibit or activate apoptosis, thus influencing treatment efficacy. PIs, in particular, modulate apoptosis by preventing the opening of the mitochondrial membrane in animal models [29, 30] and have been associated with lower rates of CD4 T cell apoptosis in HIV-infected patients [31–34]. However, these data remain controversial [35, 36]. Surprisingly, in our study, patients who received a PI-based regimen showed higher apoptotic activity. This apparent paradox could be explained by the fact that these patients had a significantly worse clinical condition (including predictors of discordant immune

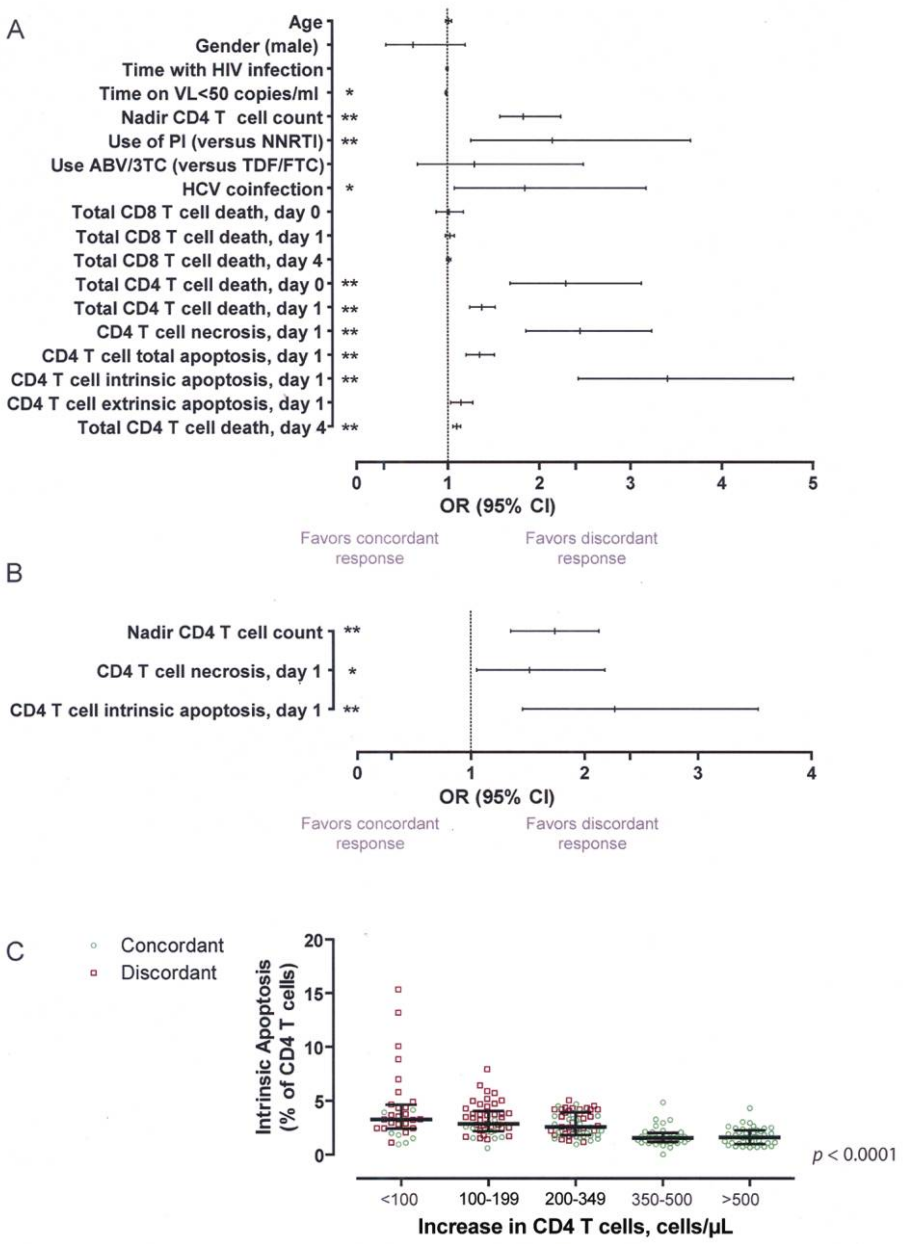

Figure 2. Predictive factors for unsatisfactory immune recovery. The ability of the indicated parameters to predict the probability of discordance was assessed in *A,* univariate analysis and *B,* multivariate analysis, which included most of parameters from the univariate analysis. *Points* denote odds ratios (ORs), and *lines* indicate 95% confidence intervals (CIs). ORs for nadir CD4 T cell counts were calculated for decreases of 50 cells/μL. Asterisks denote statistical significance: * $P < .05$, ** $P < .005$. ABV, abacavir; FTC, emtricitabine; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor; TDF, tenofovir; VL, viral load; 3TC, lamivudine. *C,* Patients were stratified according to their recovery of CD4 T cells, measured as the difference between their current CD4 T cell count and their nadir value. Five groups were defined by the increase in CD4 T cell count of <100 cells/μL ($n = 31$), 100–199 cells/μL ($n = 47$), 200–349 cells/μL ($n = 61$), 350–500 cells/μL ($n = 44$), and >500 cells/μL ($n = 42$). Intrinsic apoptosis of CD4 T cells after 1 day of ex vivo culture was analyzed. Dots indicate individual data; for illustrative purposes, discordant patients are shown as *red squares* and concordant patients as *green circles*. Median and interquartile range are shown for each group; the P value, calculated with the Kruskal-Wallis test, applies to differences among groups.

response, such as lower nadir CD4 T cell count, longer time of HIV infection, and higher rate of HCV coinfection) that probably led physicians to prefer a PI-containing regimen. Multivariate analysis, however, ruled out the use of a PI as a risk factor or as a protective factor for a discordant response. Similarly, no association was observed between use of abacavir-lamivudine or tenofovir-emtricitabine and immune response. Given these results, it seems reasonable that there are no apparent differences

between the most common antiretroviral combinations in terms of rates of apoptosis and immune response.

Many clinical factors have been related to immune recovery [5]. Our analysis demonstrates that low nadir CD4 T cell count remains the strongest factor associated with a discordant immune response and was firmly associated with immune activation and rates of intrinsic apoptosis. However, immune response was not significantly related to other parameters (eg, age, time with viral replication, or presence of HCV infection). Particularly, HCV coinfection, whose effect on immune recovery is controversial [5, 15], was not relevant in our multivariate analysis. Therefore, our data suggest that an early initiation of antiretroviral therapy in HIV-infected patients is a feasible intervention to prevent the immune deterioration that leads to unsatisfactory immune recovery.

In summary, our experimental data establish that CD4 T cell hyperactivation and the intrinsic pathway of cell death represent the determinant and final mechanisms of the unsatisfactory immune recovery in discordant patients and should be targeted to better manage treatment of these patients with more appropriate strategies. We believe that essential clinical implications of HIV infection could emerge from our data to help address such relevant decisions as when to start antiretroviral therapy and what specific therapy to choose. Because nadir CD4 T cell count was the main predictive factor of immune response and because current antiretroviral combinations had similar effects on immune recovery, we believe that HAART should be initiated earlier and that any common HAART combination can be used. Although this was a large cohort, our data arise from a cross-sectional study. Therefore, these results should be supported by further studies designed for this purpose.

Acknowledgments

This work is part of the PhD thesis of M.M. at Pompeu Fabra University, Barcelona, Spain.

Financial support. Gilead Sciences; Merck; the Spanish Foundation for AIDS Research and Prevention (FIPSE) project 06/3600; the Spanish AIDS network, Red Temática Cooperativa de Investigación en SIDA (RD06/0006); the European AIDS Treatment Network (NEAT) (to E.N. and J.B.); the Spanish Health Institute Carlos III (ISCIII) and the Health Department of the Catalan Government (Generalitat de Catalunya) (to J.B.); Generalitat de Catalunya and European Social Fund (to M.M.); and Lluita contra la SIDA Foundation (to J.R.S.).

Potential conflicts of interest. E.N., J.M., and B.C. have received research funding from Merck Sharp & Domme and have received honoraria for speaking and serving on advisory boards from Abbott, Bristol-Myers Squibb, Boehringer-Ingelheim, Gilead Sciences, GlaxoSmithKline, Roche, Janssen-Cilag, and Merck Sharp & Domme. J.B. has received honoraria as a consultant for GlaxoSmithKline. All other authors: no conflicts.

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