TESIS DOCTORAL

FACTOR DE NECROSIS TUMORAL ALFA Y VIH: IMPLICACION EN LA EVOLUCION NATURAL DE LA ENFERMEDAD Y EN LA LIPODISTROFIA ASOCIADA AL VIH Y AL TRATAMIENTO ANTIRRETROVIRAL

Sergio Veloso Esteban

UNIVERSITAT ROVIRA I VIRGILI

Departament de Medicina i Cirurgia

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Dirigida por los Profesores Dr. C Richart Jurado y Dr. F. Vidal i Marsal

Departament de Medicina i Cirurgia, Facultat de Medicina i Ciències de la Salut,

Universitat Rovira i Virgili y Hospital Universitari de Tarragona Joan XXIII

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Cristóbal Richart Jurado, Jefe de Servicio de Medicina Interna del Hospital Universitari de Tarragona Joan XXIII y Catedrático de Medicina de la Universitat Rovira i Virgili de Tarragona, y Francesc Vidal i Marsal, Jefe Clínico del Servicio de Medicina Interna del Hospital Universitari de Tarragona Joan XXIII y Catedrático de Medicina de la Universitat Rovira i Virgili de Tarragona,

Certifican que:

La tesis titulada "FACTOR DE NECROSIS TUMORAL ALFA Y VIH: IMPLICACION EN LA EVOLUCION NATURAL DE LA ENFERMEDAD Y EN LA LIPODISTROFIA ASOCIADA AL VIH Y AL TRATAMIENTO ANTIRRETROVIRAL" presentada por Sergio Veloso Esteban ha sido realizada bajo nuestra dirección y cumple los requisitos necesarios para optar al título de Doctor en Medicina

Y para que así conste y tenga los efectos oportunos, firmamos el presente documento

Tarragona, abril de 2013

Cristobal Richart Jurado   Francesc Vidal i Marsal

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Is a dream a lie if it don't come true?

(Bruce Springsteen)

A. ABREVIATURAS

(Por orden alfabético)

3TC: Lamivudina

ABC: Abacavir

ADN: Ácido DesoxirriboNucléico

ADNmt: Ácido Desoxirribonucléico mitocondrial

AN: Inhibidores de la retrotranscriptasa inversa Análogos de Nucleósidos

ARN: Ácido Ribonucléico

ATP: Adesonina Trifosfato

AZT: Zidovudina

C/EBP α / β: CCAAT-enhancer binding proteins α y β.

CD4: Linfocitos T helper CD4+

CMV: Citomegalovirus

CV: Carga viral

d4T: Estavudina

ddC: Zalcitabina

ddI: Didanosina

EFV: Efavirenz

FAPV: Amprenavir

FDA: Food & drug administration

FTC: Emtricitabina

GHRF: Factor liberador de la hormona de crecimiento

HLA: Human Leukocyte Antigen

ICAM-1: Intercellular adhesion molecule-1

IDV: Indinavir

IP: Inhibidores de Proteasa

LD: Síndrome de Lipodistrofia

LTR: Long Terminal Repeat

LPV: Lopinavir

LTNP: Long Term Non Progressor

MAI: Mycobacterium Avium Intracellulare

MCP-1: Monocyte chemotactic protein-1

MOMA-2: Monocyte macrophage 2

mmol: MiliMoles

NAN: Inhibidores de la retrotranscriptasa No Análogos de Nucleósidos

nm: Nanómetros

PBMC: Peripheral blood mononuclear cells

PPAR-γ: Peroxisome proliferator activated receptor gamma

RTV: Ritonavir

SNC: Sistema Nervioso Central

SNP: Single Nucleotid Polymorphism

SIDA: Síndrome de Inmunodeficiencia Adquirida

SREBP-1: Sterol regulatory element binding proteín-1

TARGA: Tratamiento Antirretroviral de Gran Actividad

TAS: Tejido adiposo subcutáneo

TI: Transcriptasa Inversa

TDF: Tenofovir

TNF-α: Factor de Necrosis Tumoral Alfa

TNF-R: Receptores del TNF-α

UDVP: Usuarios de Drogas por Vía Parenteral

VCAM-1: Vascular cell adhesión molecule-1

VHB: Virus de la Hepatitis B

VHC: Virus de la Hepatitis C

VHS: Virus del Herpes Simple

VIH: Virus de la Inmunodeficiencia Humana

VIS cpz: Virus de la Inmunodeficiencia Simia (chimpacé)

μl: Microlitro

ÍNDICE

A. INTRODUCCION

1. INFECCION POR VIH.

1.1. Visión histórica de la infección por VIH

Los primeros casos de infección por el virus de la inmunodeficiencia humana (VIH) fueron diagnosticados en el año 1981 cuando 5 varones jóvenes homosexuales presentaron un cuadro de neumonía producida por un germen oportunista llamado *Pneumocystis Carinii* (actualmente *Pneumocystis Jiroveci*) y una inmunodeficiencia asociada de causa no aclarada entonces[1]. Durante los meses posteriores, el número de casos similares y de otras enfermedades oportunistas se multiplicó de manera espectacular y fue a finales de ese mismo año, cuando la prestigiosa revista médica New England Journal of Medicine se hizo eco de un nuevo síndrome de inmunodeficiencia adquirida[2]. Esta inmunodeficiencia se atribuiría posteriormente al VIH y se denominaría *síndrome de inmunodeficiencia adquirida (SIDA)*.

En 1983, se aísla el VIH en una adenopatía de un paciente[3] y en 1985 se dispone de una determinación analítica para detectar el virus[4].

Hasta 1987 no se empieza a utilizar el primer fármaco antirretroviral, un inhibidor de la retrotranscriptasa análogo de nucleósidos (AN), la zidovudina (AZT). A pesar del éxito inicial, sobretodo en la disminución de la transmisión vertical del VIH[5], pronto se vio que el efecto beneficioso del AZT era transitorio y presentaba importantes efectos secundarios, sobre todo hematológicos. Hay que esperar a la primera mitad de los años 90 en que aparecen nuevos AN como zalcitabina (ddC), didanosina (ddI), estavudina (d4T) y lamivudina (3TC) para iniciar la llamada biterapia[6], combinación de 2 fármacos que, sin embargo, tampoco aportó grandes éxitos en el tratamiento del VIH.

En 1996 se comercializa una nueva familia de fármacos, los Inhibidores de Proteasa (IP) y se inicia la triple terapia o tratamiento antirretroviral de gran actividad[7] (TARGA).

El TARGA, asociación de 2 AN y 1 IP, conseguía controlar la infección, negativizar la carga viral (CV), recuperar la función inmunológica y disminuir las infecciones oportunistas y la mortalidad[8].

La parte negativa de esta estrategia terapéutica era la gran cantidad de pastillas a tomar y los efectos secundarios.

En los últimos 10 años se han comercializado nuevos fármacos antirretrovirales. Disponemos actualmente de casi una treintena de ellos, que podemos utilizar en diferentes combinaciones, con un número menor de comprimidos (1-4 al día), muchos de ellos en dosis única diaria y con mejor tolerancia por parte del paciente.

En resumen, durante estos 30 años de pandemia por el VIH, hemos pasado de una primera etapa en que la infección por VIH era una enfermedad intratable y mortal a conseguir que se convierta en una enfermedad crónica controlada con medicación.

Sin embargo, no somos todavía capaces de eliminar por completo el VIH del organismo. Esto obliga a mantener indefinidamente el TARGA, circunstancia que ha hecho aparecer nuevos problemas en el manejo clínico de estos pacientes. Problemas como la resistencia del VIH a los antirretrovirales y la aparición de efectos secundarios tardíos del tratamiento como las alteraciones metabólicas y el síndrome de redistribución grasa, también conocido como lipodistrofia (LD), son habituales hoy en día y dificultan el manejo de los pacientes infectados.

1.2. Epidemiología del VIH

Epidemiología a escala mundial

Se han descrito casos de infección por el VIH en prácticamente todos los países del mundo. Según los últimos datos del Joint United Nations Programme on HIV/AIDS (UNAIDS), a finales de 2010 existían unos 34.000.000 de personas con la infección por VIH.

La distribución por zonas (figura 1) muestra que más de 2/3 de las infecciones se producen en países no desarrollados, sobretodo en África subsahariana.

De manera global, un 80% de las infecciones por VIH se han contraído por vía sexual, siendo un 75% de las mismas por contacto heterosexual.

Adults and children estimated to be living with HIV | 2010

Total: 34.0 million [31.6 million – 35.2 million]
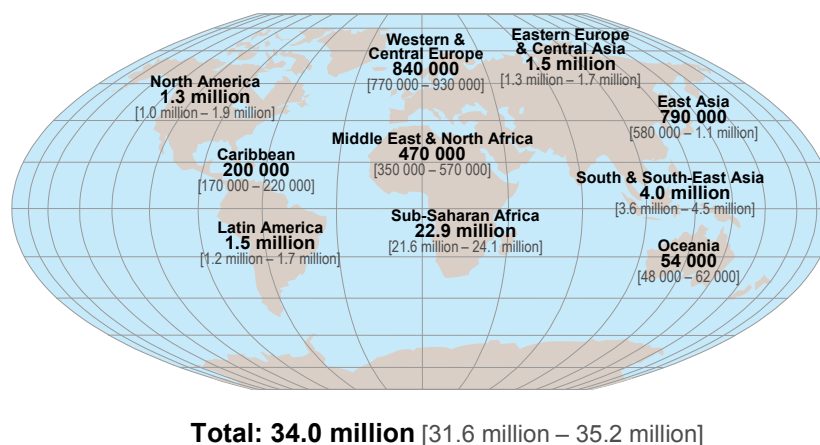
Figura 1. Distribución geográfica del número estimado de personas infectadas por el VIH a finales del año 2010. Extraido de ONUSIDA: www.unaids.org.

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En 2011, el número total de casos de SIDA diagnosticados en España era de 81.855 según el Registro Nacional de SIDA. En 2011, se diagnosticaron 1038 nuevos casos de SIDA y 2763 de nuevas infecciones por VIH (figura 2). De éstas últimas, el 85% fue por vía sexual (54% homosexual y 31% heterosexual) y sólo el 5% fueron usuarios a drogas por vía parenteral. El porcentaje de nuevos casos en personas extranjeras subió hasta el 37%, un 6% más que el año anterior. El 46% del global de los nuevos diagnósticos presentaron diagnóstico tardío, siendo la tuberculosis la enfermedad oportunista más frecuente, seguida por la neumonía por *Pn. Jirovecii* y la candidiasis esofágica.
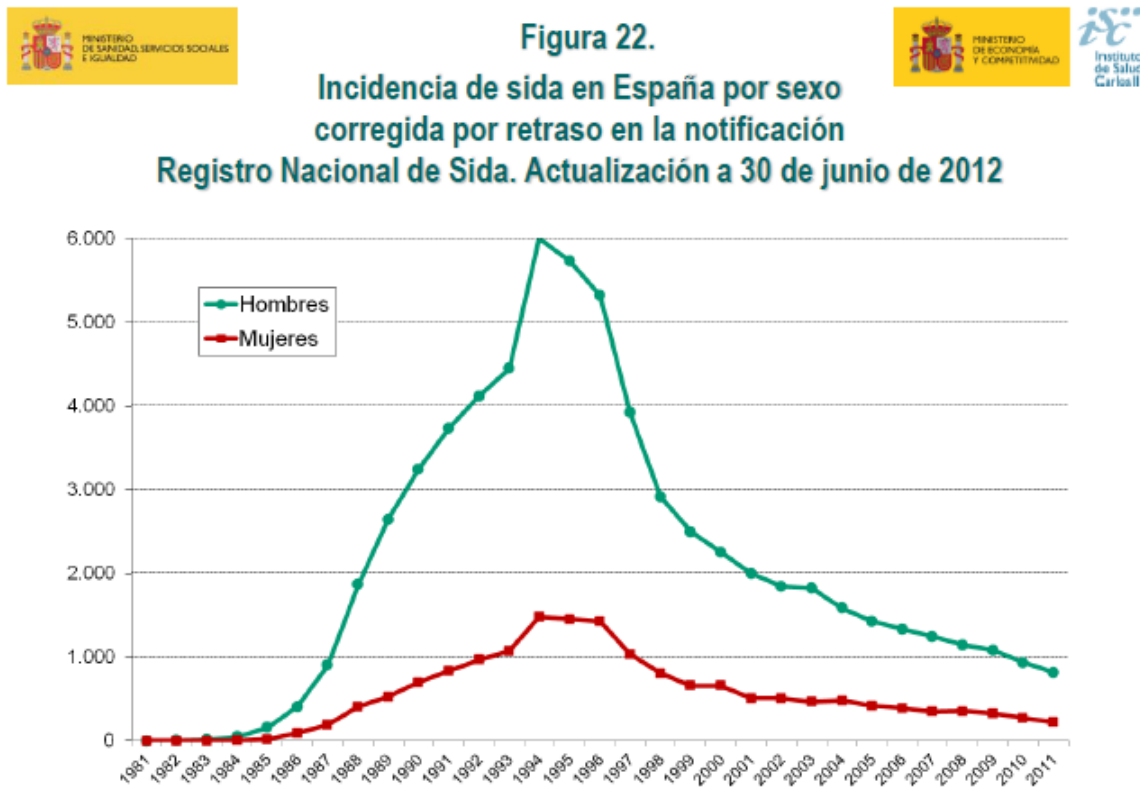
Figura 2: Incidencia anual de SIDA en España corregida por retraso en la notificación. Registro Nacional del SIDA. Actualización a 30 de junio de 2012. Extraída de: www.msc.es

1.3. Etiopatogenia de la infección por VIH

El VIH pertenece a la familia de los retrovirus, virus con el material genético en forma de ácido ribonucleico (ARN), capaces de transformarlo en ácido desoxirribonucleico (ADN) proviral mediante un enzima, la Transcriptasa Inversa (TI). El ADN proviral es integrado posteriormente en el ADN de la célula huesped[9]. Pertenece al género Lentivirus, virus que no inducen transformación tumoral pero si un efecto citopático lento en las células que infectan. Existen 2 tipos de VIH, el VIH-1 y VIH-2. En el presente trabajo nos referiremos siempre al VIH-1, excepto si se indica lo contrario.

El VIH tiene un origen zoonótico. Proviene de un virus similar, el virus de la inmunodeficiencia simia (VIS), que afecta a determinadas especies de chimpancés. Estudios filogenéticos demuestran que la transmisión ínter-especies (de primates a hombre) tuvo lugar a principios del siglo XX[10].

1.3.1. Estructura del VIH

El VIH es una partícula esférica de 80 a 100 nm, con una estructura en 3 capas (figura 3):

1. Capa externa o envoltura: Formada por una bicapa lipídica, antígenos de histocompatibilidad de clase I y II, y la glicoproteína viral gp 160 con sus 2 dominios, gp 120 y gp 41.
2. Cápside: Formada por diferentes proteínas, como la p17 y la p24. En ella se aloja el material genético viral.
3. Capa interna o nucleóide: Contiene dos hebras completas del genoma viral y las enzimas virales implicadas en el ciclo replicativo del VIH: la TI, la integrasa y la proteasa.

El genoma del virus es un ARN de cadena única formada por 2 hebras idénticas de polaridad positiva y de 9,6 kilobases de longitud. Está constituido por 9 genes, 3 estructurales (gag, pol y env) y 6 adicionales que codifican para proteínas reguladoras y accesorias (tat, rev, nef, vif, vpr y vpu)[11].

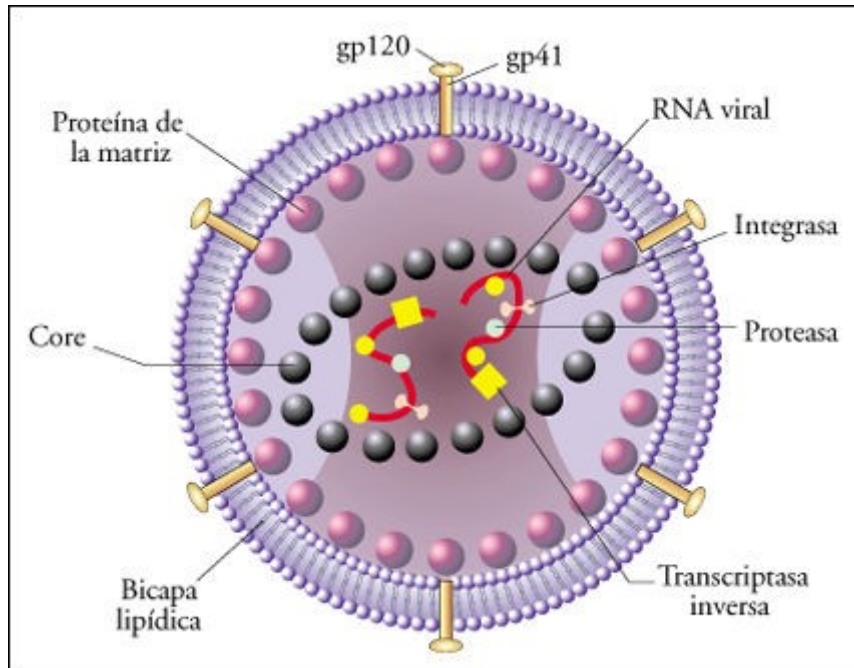**Figura 3**. Estructura del VIH. Extraida de: www.cobach-elr.com

1.3.2. Ciclo biológico del VIH

El ciclo biológico del VIH puede dividirse en diferentes etapas (figura 4).

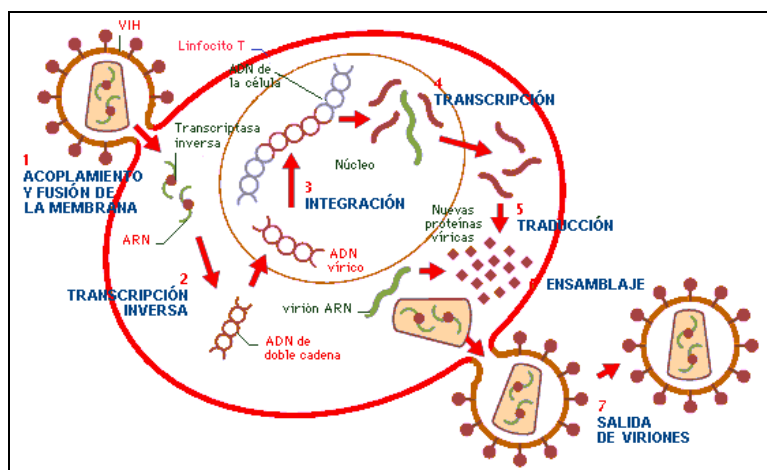**Figura 4**. Ciclo replicativo del VIH. Extraida de: VIH y SIDA. http://www.ctv.es

La primera de ellas es la fusión e internalización del virus a través de la membrana celular utilizando el receptor CD4 y los correceptores CCR5 y/o CXCR4[12]. En el interior celular, la TI transforma el ARN en ADN. La doble cadena de ADN es transportada al núcleo celular por el complejo de integración y la integrasa del VIH regula el proceso de integración en el ADN nuclear. A partir de aquí, el virus utilizará la maquinaria celular para sintetizar ARN genómico y mensajero para la síntesis de las proteínas virales y formación de nuevos viriones que abandonarán la célula por gemación.

1.3.3. Inmunopatogenia e historia natural de la infección por VIH

Inmunopatogenia

El VIH infecta a células que expresan el receptor CD4, mayoritariamente linfocitos T colaboradores y células del sistema mononuclear-fagocítico. La diana principal son los linfocitos CD4 y su destrucción progresiva va a ser el evento principal en la infección por VIH.

El principal mecanismo de pérdida linfocitaria es por efecto citopático directo debido a la replicación viral, aunque existen mecanismos indirectos. Entre estos últimos, destacan los relacionados con la propia respuesta inmune (los linfocitos infectados expresan proteínas virales y son destruidos por el propio sistema inmune) y a fenómenos de apoptosis linfocitaria[13].

La respuesta inmunitaria frente al VIH es doble: humoral y celular. La respuesta humoral es muy intensa pero tiene una limitada capacidad neutralizante. La respuesta celular (linfocitos CD4 colaboradores y CD8 citotóxicos) es importante para controlar la replicación viral en fases iniciales. Desafortunadamente, la eficacia de ambos tipos de respuesta es limitada en el tiempo y se llega a la fase final de la infección con deterioro inmunológico y replicación viral acelerada[14].

Historia natural de la infección por VIH

La infección por el VIH se puede dividir en 3 fases evolutivas. (figura 5)

From **Immunity: The Immune Response in Infectious and Inflammatory Disease**
by DeFranco, Locksley and Robertson
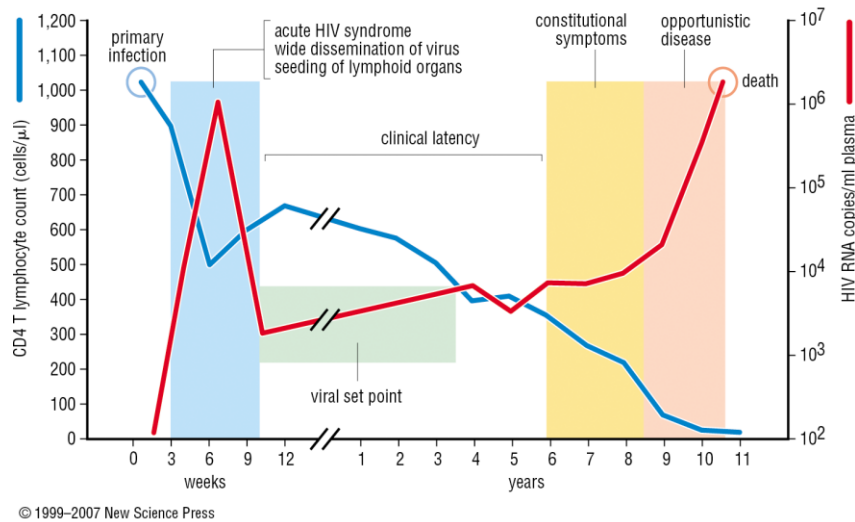
Figura 5. Historia natural de la infección por VIH. Extraido de DeFranco,
Locksley and Robertson (2007). Inmunity: The immune response in
infectious and inflammatory disease Oxford University Press. UK

1. Fase aguda

Tiene lugar durante las primeras semanas de la infección. Hasta un 50% de los pacientes
están asintomáticos. Existe un descenso de los linfocitos T CD4+, tanto por destrucción
directa como por redistribución periférica[15]. Se acompaña de una gran replicación y
diseminación viral, sobretodo a nivel del tejido linfático y del sistema nervioso central. En
este último, persistirá replicación de manera continua, incluso en pacientes que reciben
TARGA. La presencia de estos reservorios explica, al menos en parte, la incapacidad del
TARGA para erradicar completamente la infección[16].

2. Fase crónica

Se consigue un equilibrio entre la replicación viral y la respuesta inmunitaria, manteniendo al
paciente en fase de latencia clínica a pesar de la replicación del VIH, sobretodo en órganos
linfoides[17].

El descenso conseguido en la carga viral (set point viral) al principio de esta fase va a ser uno de los factores predictivos más importante en la evolución de la enfermedad a SIDA[18]. En función de la velocidad de progresión a SIDA, podemos diferenciar 3 grupos de pacientes:

A. Progresores rápidos (5-10%): Progresión a SIDA entre 1 y 5 años. Pacientes infectados por fenotipos virales más agresivos de VIH[19] y que no logran un buen control de la CV al principio de la infección.

B. Progresores típicos (80-90%): Progresión a SIDA en torno a los 10 años de la infección[20]. Buen control inicial de la CV pero con posterior caída progresiva de los linfocitos CD4.

C. Progresores lentos (1-5%): Pacientes que tras más de 10 años de infección permanecen asintomáticos, con niveles de CD4 superiores a 500 cel/ul y CV muy baja. Son llamados "no progresadores" o "progresadores lentos"[21] (long term non progressors o LTNP). Dentro de este grupo existe un subgrupo llamado "controladores de élite" que presentan una CV indetectable en el tiempo. Son menos del 1% del total[22].

3. Fase final

Aparición del SIDA. Descenso de los linfocitos CD4 por debajo de 200 células/µl, aumento de la CV y aparición de infecciones oportunistas y/o neoplasias con elevada morbi-mortalidad.

1.3.4. Determinantes genéticos en la inmunopatogenia y la historia natural de la infección por VIH. Papel del TNF-α

El curso variable de la infección por VIH viene determinada por varios factores. De los relacionados con el huésped, destacan los siguientes:

1. _El complejo mayor de histocompatibilidad (HLA):_ El HLA-B35 parece relacionarse con mayor progresión de la enfermedad[23] y el B57 con un mejor control de la viremia[24].

2. *Receptores de quimiocinas*: Su identificación en 1996[25] como co-receptores necesarios para la entrada del VIH a la célula mejoró nuestro conocimiento sobre los factores genéticos que afectan, tanto a la susceptibilidad como al grado de progresión de la infección. La más estudiada y conocida es la deleción de 32 pares de bases en el gen que codifica para el co-receptor CCR5 (CCR5-Δ32)[26]. Los pacientes heterocigotos presentan una progresión más lenta de la enfermedad[27] y los homocigotos tienen menor susceptibilidad a la infección[28], aunque pueden infectarse por cepas CXCR4. Es conocido el caso del "paciente Berlin"[29]. Se trata de un paciente infectado por el VIH con una leucemia mieloide aguda al que se realizó un trasplante de células madres de un donante compatible CCR5Δ32/Δ32 y que más de 3 años después permanece con carga viral indetectable sin realizar ningún tipo de TARGA.

3. *Las citoquinas*, polipéptidos secretados por células del sistema inmune, juegan un papel importante en la regulación de la replicación viral. El factor de necrosis tumoral alfa (TNF-α), interleukina-1 y 6 son citoquinas proinflamatorias que se encuentran elevadas en pacientes infectados por el VIH y que favorecen la replicación viral[30].

Dentro de la disregulación inmune que tiene lugar en la infección por el VIH, el TNF-α juega un papel principal. Favorece la replicación viral en células infectadas y modula el fenómeno de apoptosis de los linfocitos CD4+[31].

En fases iniciales de la infección, proteínas virales como Nef, Vpr y Tat, mimetizan la acción del TNF-α. Inhiben la apoptosis celular de las células infectadas[31,32] y favorecen la replicación viral estimulando la actividad de los LTR (long term repeat) virales, mediante el factor de transcripción Kappa beta (NF-κB)[33], entre otros activadores.

En fases más avanzadas, con niveles de TNF-α más elevados, las proteínas virales potencian la acción apoptótica mediada por los receptores del TNF. Además, Nef previene la apoptosis de las células infectadas al interferir en la muerte celular mediada por la vía del TNF-R al inhibir ASK-1 y la activación de las caspasas 3 y 8[34].

La producción de TNF-α está, al menos parcialmente, determinada genéticamente[35]. Se han identificado varios polimorfismos de nucleótido simple (SNP) en el gen del TNF-α. Los más estudiados son en la posición -238 (*G>A*), -308 (*G>A*), -863 (*C>A*)[36].

La información disponible hasta el momento respecto al papel del TNF-α en la vulnerabilidad y la velocidad de progresión de la infección por VIH es inconsistente. Existen estudios que encuentran asociación[37,38], hallazgo que otros estudios no han confirmado[39,40].

2. TRATAMIENTO ANTIRRETROVIRAL DE GRAN ACTIVIDAD (TARGA)

2.1. Fármacos antirretrovirales

Desde la introducción en 1987 del AZT como primer fármaco antirretroviral, los cambios en el tratamiento de la infección por VIH han sido vertiginosos. Actualmente disponemos de casi una treintena de antirretrovirales que han permitido individualizar y simplificar el tratamiento de estos pacientes, tanto en número de pastillas como en tomas.

Los fármacos antirretrovirales actúan en diferentes momentos del ciclo replicativo viral. (figura 6)
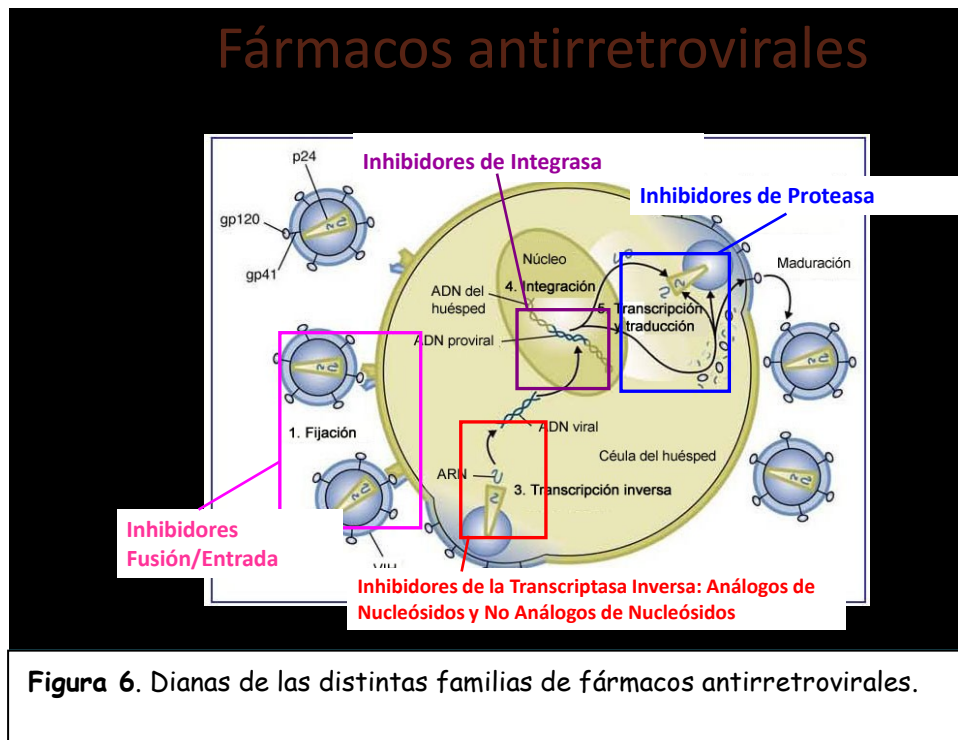
Figura 6. Dianas de las distintas familias de fármacos antirretrovirales.

Los podemos clasificar en diferentes familias de acuerdo con la diana terapéutica sobre la que actúan (tabla 1):

AN	ANt	NAN	I.P	I.F	I.I	I. CCR5
Zidovudina	Tenofovir	Efavirenz	Indinavir	Enfuvirtida	Raltegravir	Maraviroc
Didanosina		Nevirapina	Saquinavir			
Zalcitabina		Delarvidina	Ritonavir			
Estavudina		Etravirina	Nelfinavir			
Lamivudina			Lopinavir			
Abacavir			Fosamprenavir			
Emtricitabina			Atazanavir			
			Darunavir			

Tabla 1. Fármacos antirretrovirales

AN: Inhibidores de la TI análogos de nucleósidos, ANt: Inhibidores de la TI análogos de nucleótidos, NAN: Inhibidores de la TI no análogos de nucleósidos, I.P: Inhibidores de proteasa, I.F: Inhibidores de fusión, I.I: Inhibidores de la Integrasa, I. CCR: Inhibidores del coreceptor CCR5

1. *Inhibidores de la transcriptasa inversa (TI)*: Se dividen en AN, análogos de nucleótidos (ANt) y no análogos de nucleósidos (NAN). Los AN y ANt funcionan como nucleótidos naturales que, al incorporarse a la cadena de ADN bloquean la continuación del proceso. Los NAN inhiben directamente a la TI uniéndose a ella.

2. *Inhibidores de proteasa (IP):* Bloquean las proteasas virales.

3. *Inhibidores de la fusión (IF):* Bloquean la acción de la gp41, evitando la fusión viral.

4. *Inhibidores de la integrasa (II):* Impiden la unión del ADN proviral con el ADN celular

5. *Inhibidores del co-receptor CCR5:* Impiden la internalización del virus bloqueando el correceptor CCR5.

2.2. Inicio del tratamiento antirretroviral. Manejo clínico

El TARGA es, en la actualidad, el "gold standard" del tratamiento de la infección por VIH[41]. Se basa en el uso de, al menos, 3 fármacos para controlar la replicación viral. Diferentes guías orientan acerca de cuando y como iniciar y cambiar el tratamiento antirretroviral[42,43,44].

En general, se inicia el TARGA con 2 fármacos AN junto con un tercer fármaco, que puede ser un NAN, un IP potenciado con ritonavir o un inhibidor de la integrasa.

Actualmente, y de manera resumida, las recomendaciones de las guías para iniciar tratamiento son:

1. Iniciar TARGA en pacientes sintomáticos y, con ciertas diferencias en la fuerza de la recomendación, en pacientes asintomáticos con cifras de CD4 inferior a 500 cel/μl .

2. Independientemente del número de CD4 en situaciones clínicas especiales: Coinfección con VHC y/o VHB con criterios de tratamiento, nefropatía por VIH, CV elevada, edad superior a 55 años, riesgo cardiovascular elevado, mujeres seropositivas embarazadas y parejas serodiscordantes.

El control se realiza, habitualmente, de forma ambulatoria con seguimiento de la CV, que debe negativizarse, y de los CD4. Es importante la necesidad de realizar correctamente el tratamiento, ya que una mala adherencia puede provocar la selección de cepas de VIH mutantes resistentes y obligar a cambiar a pautas de TARGA más complejas.

A pesar de los conocimientos y avances actuales, hoy día no es posible la erradicación del VIH, por lo que el objetivo primordial del TARGA es controlar y cronificar la infección. Este último punto puede ser considerado como conseguido en la actualidad[45]. Sin embargo, la cronificación y el uso cada vez más temprano del TARGA se asocia a la aparición de resistencias al tratamiento en pacientes con falta de adherencia y, sobre todo, la aparición de diferentes efectos secundarios.

2.3. Efectos secundarios del TARGA

Los efectos secundarios del TARGA, independientemente de su repercusión clínica, contribuyen a empeorar la calidad de vida del paciente y a dificultar el cumplimiento terapéutico. La toxicidad causada por los antirretrovirales es un problema creciente debido al mayor número de pacientes que reciben tratamiento, a su mayor supervivencia y al carácter indefinido del tratamiento.

Son la principal causa de modificación y/o abandono del TARGA. Se estima que la probabilidad de abandonar un primer o segundo TARGA durante el primer año por una toxicidad precoz asociada a los ARV es de alrededor de un 25 % y la aparición de efectos adversos a largo plazo (acidosis láctica, LD, desmineralización ósea…) oscila entre el 25-50% de los pacientes, provocando una disminución de la calidad de vida[46].

En la tabla 2 se muestran los efectos secundarios más importantes.

---

1. Toxicidad mitocondrial

2. Hepatotoxicidad

3. Reacciones de hipersensibilidad

4. Síndrome de reconstitución inmune

5. Alteraciones del metabolismo óseo

6. Alteraciones morfológicas: Síndrome de redistribución grasa o lipodistrofia

7. Alteraciones metabólicas: Dislipemia, resistencia a la insulina y diabetes

---

Tabla 2. Efectos secundarios del TARGA

A continuación se describen con más detalle la toxicidad mitocondrial y las alteraciones y morfológicas, pues son objeto de estudio en la presente tesis.

1. Toxicidad mitocondrial

La principal toxicidad de los fármacos AN se debe a la inhibición de la ADN polimerasa mitocondrial gamma[47,48]. Este efecto es conocido desde la monoterapia con AZT con el clásico cuadro de miopatía asociada al AZT[49].

Sin embargo, no todos los AN afectan de igual manera a la función mitocondrial. Existe un orden decreciente de toxicidad, siendo los dideoxinucleótidos los más tóxicos y ABC el menos lesivo a nivel mitocondrial: ddC > ddI > D4T > AZT > 3TC > ABC[50].

Las manifestaciones clínicas de la toxicidad mitocondrial son variadas y dependen del órgano afectado. Suele afectar a tejidos muy dependientes de la función mitocondrial como el músculo esquelético o el corazón, pero también afecta a tejidos en los que el papel de la mitocondria en su homeostasis es aparentemente menos relevante como en el tejido adiposo, donde la toxicidad mitocondrial se ha relacionado con la LD que se observa en determinados pacientes, hecho que se comentará con mayor profundidad más adelante en esta Tesis. Otros ejemplos de toxicidad mitocondrial son la polineuropatia por d4T, la pancreatitis por ddI o la acidosis láctica.

2. Alteraciones morfológicas

Aparecieron al poco tiempo de empezar a utilizarse el TARGA. Se le llamó, inicialmente, *síndrome de lipodistrofia asociado al VIH*. Actualmente, también se utiliza el término de *síndrome de redistribución grasa*. A continuación se tratará con mayor profundidad dicho síndrome, que es la base de la actual tesis.

3. SINDROME DE LIPODISTROFIA ASOCIADA AL VIH / TARGA

3.1. Introducción

Los primeros casos de LD en pacientes VIH+ tratados con TARGA se describieron en 1998 coincidiendo con la introducción de los IP. Esta relación temporal hizo que se atribuyera, de manera errónea, la LD al uso de IP[51,52]. Posteriormente, se describieron casos con otros antirretrovirales distintos a los IP[53]. El nombre del síndrome tiene su origen en la similitud fenotípica con las lipodistrofias congénitas, enfermedades que presentan pérdidas selectivas del tejido adiposo[54].

Tras más de 10 años de estudio, sigue sin conocerse totalmente la etiopatogenia de la LD[55] y, actualmente, se considera un síndrome multifactorial en el que intervienen diversos factores[56]:

1. El propio VIH provoca un fenómeno inflamatorio, tanto a nivel sistémico como local en el tejido adiposo
2. Los fármacos antirretrovirales tienen efectos secundarios a nivel del tejido adiposo.
3. El huésped, mediante una probable vulnerabilidad determinada genéticamente.

Además del problema estético y la alteración de la calidad de vida por la afectación psicológica que provoca el cambio del aspecto corporal[57], la LD puede inducir a una peor adherencia al tratamiento con el consiguiente riesgo de aparición de resistencias. Así mismo, están descritas una serie de asociaciones con implicación clínica como la aparición de un síndrome metabólico (hiperlipidemia, insulin-resistencia y diabetes), enfermedad cardiovascular y osteoporosis[58,59,60].

3.2. Definición

La LD consiste en la aparición de cambios en la distribución de la grasa corporal. Éstos producen 3 fenotipos diferentes y característicos[55]. En la figura 7[61] se muestran estos fenotipos:
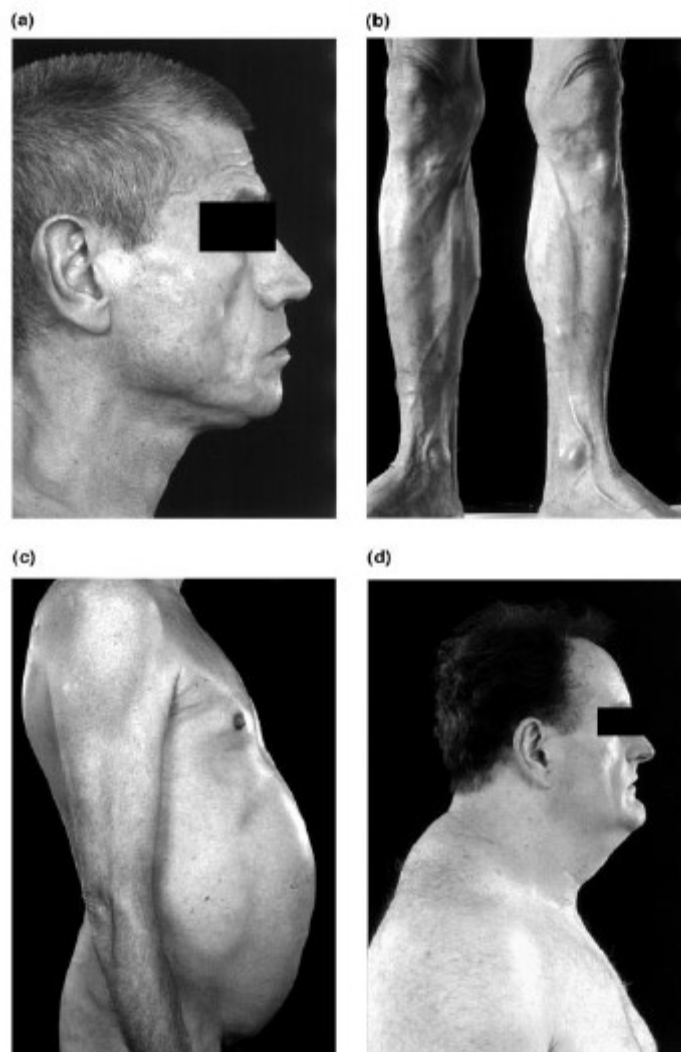
Figura 7. Lipoatrofia (a y b). Lipohipertrofia (c y d). Extraida de Mallon PWG et al. HIV Medicine 2001; 2:166-173

1. LIPOATROFIA: Pérdida de la grasa subcutánea en cara, nalgas y extremidades (alteración predominante en la LD asociada a VIH[62])

2. LIPOHIPERTROFIA: Acumulación de grasa visceral a nivel abdominal, región dorso-cervical y mamas. Aparición de lipomatosis en tronco y extremidades.

3. AFECTACION MIXTA: Presencia de ambos fenotipos en el mismo paciente.

Los cambios corporales pueden acompañarse de cambios metabólicos, sobretodo si el TARGA incluye IP de primera generación[63] (ritonavir, indinavir). Entre los cambios metabólicos deben destacarse:

1. *Alteración del metabolismo de los carbohidratos*: Resistencia a la insulina y diabetes mellitus.

2. *Alteración del metabolismo lipídico*: Hipertrigliceridemia e hipercolesterolemia.

Aunque los cambios metabólicos pueden observarse en pacientes sin LD[64], es más frecuente la coexistencia de ambas complicaciones[65].

3.3. Prevalencia y factores de riesgo

La prevalencia de la LD asociada al VIH es variable en las primeras cohortes que se estudiaron[65,66,67,68,69,70]. Oscila entre el 20 y el 70% de los pacientes que reciben TARGA, aunque en la mayoría de las series se sitúa alrededor del 40-50%[71]. Influye en esta variabilidad la falta de una definición objetiva de LD[68,72]. En estudios más recientes, la prevalencia de LD permanece estable en torno al 50-60%, con descenso en la incidencia de nuevos casos[73,74,75]. De los fenotipos descritos, el estudio FRAM demuestra que en los pacientes con LD el fenotipo prevalente es la lipoatrofia, tanto en hombres como mujeres. Sin embargo, no encuentra mayor prevalencia de lipohipertrofia en los pacientes con LD que sin ella[76,77].

La mayoría de estudios coinciden en los factores de riesgo para presentar LD[78,79,80,81,82]. La tabla 3 resume todos estos factores en función de su origen[55].

Las alteraciones metabólicas están más relacionadas, en general, con el uso de IP (con la excepción de atazanavir) mientras que son menos prevalentes en los pacientes utilizan NAN o AN[71,82]. Las alteraciones del metabolismo lipídico oscilan entre el 30-80% en general. Un 40-80% presentan hipertrigliceridemia y un 10-50% hipercolesterolemia[83,84].

FACTORES DE RIESGO PARA DESARROLLAR LIPODISTROFIA

PACIENTE	Edad /Sexo / Índice de masa corporal
	Grupo étnico / Factores genéticos
VIH	Duración de la infección / Diagnostico de SIDA
	Valores de CD4 y CV
TARGA	Duración del tratamiento
	Tratamiento análogos de la timidina / IPs / NAN

Tabla 3. Factores de riesgo para desarrollar lipodistrofia. Extraída de Moreno et al. AIDS Rev 2009; 11: 126-34.

La prevalencia de alteraciones en el metabolismo carbohidratado es menor que en el metabolismo lipídico, apareciendo la diabetes mellitus clínica en un 2-5% de los casos. Sin embargo, la prevalencia de resistencia a la insulina es de hasta un 50% en pacientes con IP y de un 25% en pacientes con AN[84,85].

Estas alteraciones se pueden enmarcar dentro del síndrome metabólico (diabetes mellitus, hiperlipidemia e hipertensión arterial) ya conocido en pacientes sin infección por VIH. De hecho, este síndrome es más frecuente en pacientes VIH con TARGA que en la población general (20,8 vs 15,8%)[86]

3.4. Diagnóstico

Pasados más de 10 años desde la detección de los primeros casos, aún no existe una definición consensuada de LD[87]. En 2003, *Carr* y colaboradores propusieron una definición objetiva de LD a partir de un "score" basado en parámetros antropométricos, metabólicos y técnicas de imagen, similar a los criterios diagnósticos de las enfermedades reumatológicas[72]. Sin embargo, su uso no se ha generalizado debido a su complejidad que dificulta utilizarla en la práctica clínica diaria[88].

El método más ampliamente aceptado para diagnosticar la LD es la valoración subjetiva del paciente junto la confirmación por parte del médico de cambios físicos en una o más zonas del cuerpo a la exploración física.

Otros parámetros objetivos útiles (tabla 4), son parámetros antropométricos, perímetro abdominal y técnicas de imagen que cuantifiquen la grasa (TAC, RMN y DEXA). Sin embargo, éstas últimas, suelen utilizarse más en el ámbito investigador que en la práctica clínica debido a la no disponibilidad generalizada, su coste y el no haber demostrado claras ventajas sobre el examen físico[84].

METODOS UTILIZADOS PARA VALORACION CORPORAL EN LA LIPODISTROFIA
Medidas antropométricas
Ecografía
Impedancia bioeléctrica
Tomografía computerizada
Resonancia magnética
DEXA (Double-energy X-ray absorptiometry)

Tabla 4. Métodos más usados para diagnosticar la lipodistrofia

3.5. Etiopatogenia

La etiopatogenia de la LD en los pacientes VIH que reciben TARGA no se conoce en su totalidad. Sin embargo, si que conocemos diferentes factores que intervienen en la aparición de la misma. Se han identificado factores que dependen de los fármacos, del VIH y del propio huésped que analizaremos en detalle a continuación.

Los cambios estructurales y funcionales finales van a afectar al tejido adiposo. Es interesante, por tanto, conocer mejor la estructura y fisiología del mismo para entender mejor los cambios producidos por la LD.

3.5.1. Tejido adiposo

El tejido adiposo está formado por adipocitos que se hallan situados en un lecho estromal constituido por macrófagos, fibroblastos, células endoteliales y preadipocitos.
Además del almacenamiento de lípidos, el tejido adiposo presenta una gran actividad metabólica[89]. Sintetiza diferentes proteínas (adipocitoquinas) y hormonas que actúan como mediadores inflamatorios y del metabolismo lipídico y de los carbohidratos tanto a nivel paracrino como endocrino[90]. (figura 8)
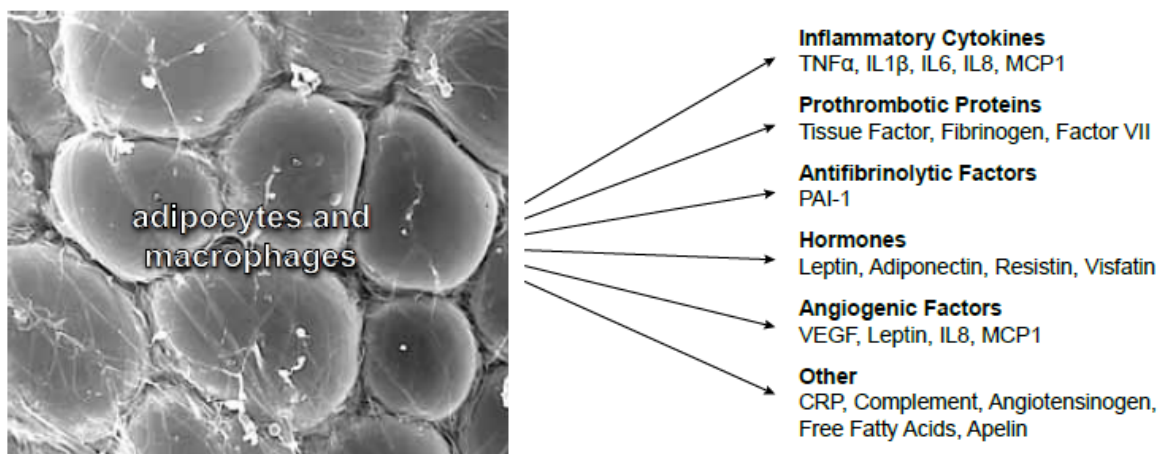
Figura 8: Sustancias secretadas por el adipocito. Extraído de Schäfer K. Hellenic J Cardiol. 2011, 52: 327-336

3.5.2. Diferenciación adipocitaria

La adipogénesis es el proceso de formación de adipocitos a partir de un precursor mesenquimal pluripotencial que se diferencia inicialmente a adipoblasto y, posteriormente, a preadipocito antes de conseguir su forma adulta[91]. Se trata de un proceso altamente dirigido y controlado por diferentes factores de transcripción adipocitarios proadipogénicos y antiadipogénicos[92] (figura 9). Entre los proadipogénicos, destacan los siguientes[93]:

1. CCAAT-enhancer binding proteins (C/EBP) α y β.
2. Sterol regulatory element binding proteín-1 (SREBP-1)
3. Peroxisome proliferator activated receptor gamma (PPAR-γ)

Por otra parte, el TNF-α es un potente agente antiadipogénico y, en condiciones homeostáticas, el efecto combinado de los factores pro y antiadipogénicos mantiene en equilibrio el proceso de adipogénesis
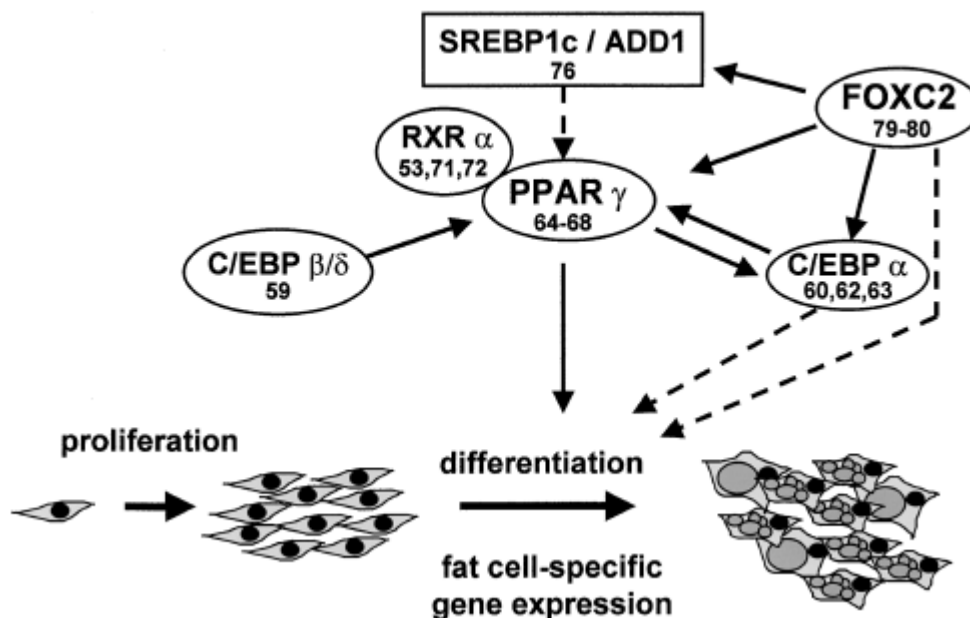
Figura 9. Diferenciación adipocitaria. Extraído de Valet P. Nature 1995; 373: 117-122.

En los pacientes infectados por el VIH con LD, el tejido adiposo presenta cambios morfológicos importantes[94,95,96]. Destacan los siguientes:

1. Disminución del número de adipocitos y anomalías en su tamaño
2. Incremento de la apoptosis adipocitaria
3. Formación de lipogranulomas focales
4. Aumento de macrófagos
5. Mayor proliferación vascular
6. Hiperplasia mitocondrial.

Las figuras 10 y 11 ilustran la diferencia a nivel microscópico entre el tejido adiposo normal y el de un paciente con LD y la presencia de apoptosis. En la apoptosis, destaca la disminución del tamaño celular, vesículas citoplasmáticas y condensación de la cromatina entre otros hallazgos (ver flechas en figura 10).
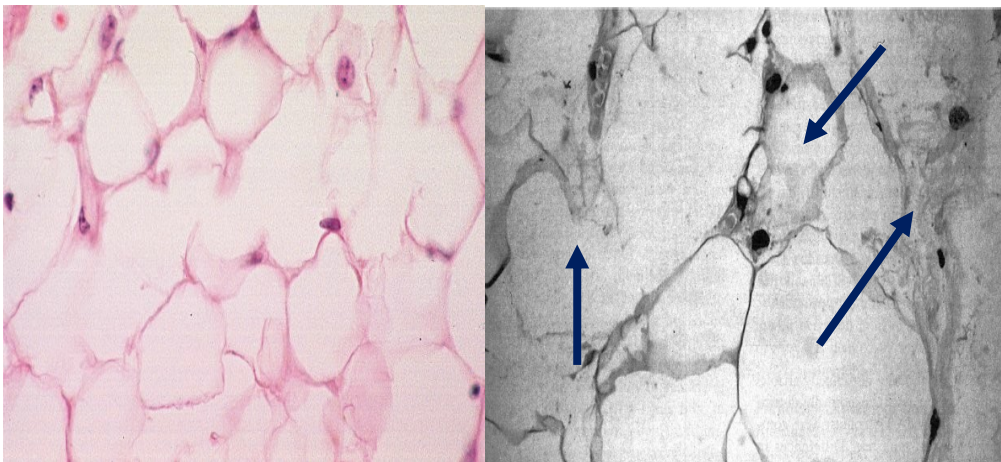
Figura 10. Diferencia entre tejido adiposo sano y en la LD asociada a VIH. Extraido de http://lahistoteca.blogspot.com.es y de Domingo P et al. AIDS 1999; 13: 2261-67.
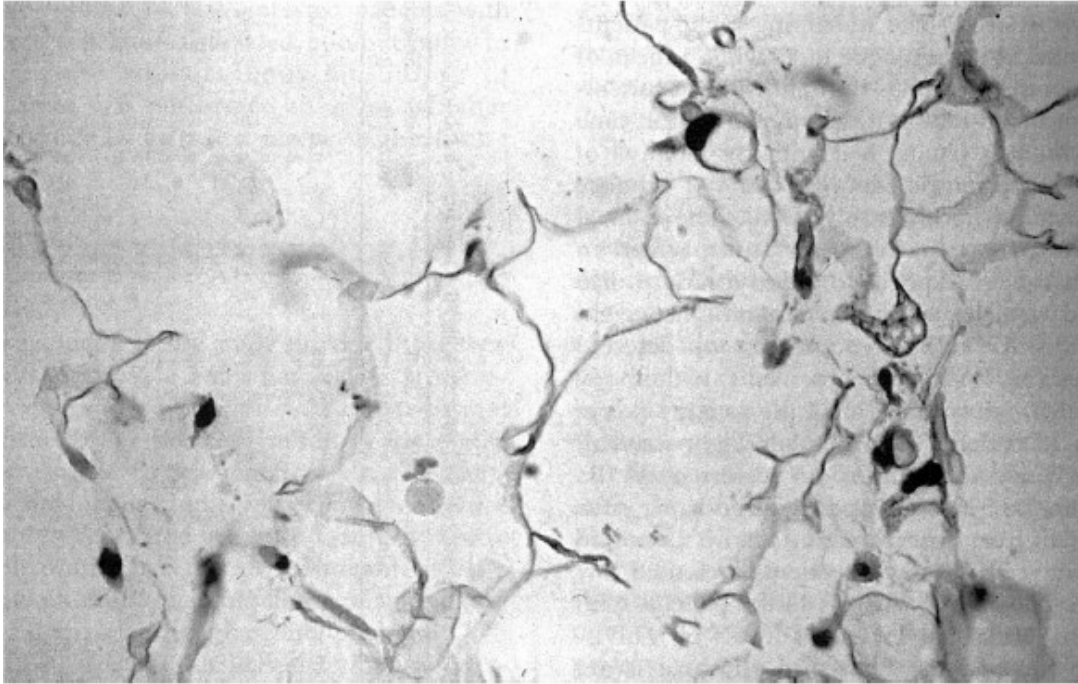
Figura 11. Apoptosis adipocitaria. Extraído de Domingo P et al. AIDS 1999; 13: 2261-67

3.5.3. Toxicidad mitocondrial

Ya se ha comentado previamente la afectación mitocondrial mediada por los fármacos antirretrovirales tipo AN mediante la inhibición de la ADN polimerasa gamma.

In vivo, el daño mitocondrial se traduce en una reducción del ADN mitocondrial (ADNmt) en diferentes tejidos, como el hepático y en células mononucleares de sangre periférica (PBMC)[97,98]. Respecto al tejido adiposo, se ha descrito en biopsias de TAS de pacientes con LD una disminución del ADNmt comparadas con biopsias de pacientes sin LD o sin tratamiento[97,99,100,101].

Mientras en el tejido adiposo queda clara la disminución del ADN mt, se ha intentado correlacionar los niveles de ADNmt en PBMC (muestra más fácil de obtener) con los niveles de ADNmt en tejido adiposo subcutáneo para utilizarlo como marcador de la toxicidad mitocondrial. Sin embargo, los niveles de ADNmt en PBMC no parecen correlacionarse bien con los del tejido adiposo[102,103,104,105,106,107].

Se ha descrito afectación mitocondrial por otras vías diferentes a la inhibición de la ADN polimerasa gamma en pacientes con niveles normales de ADNmt que presentan lactoacidosis y LD[108,109]. Destacan las siguientes:

- Daño oxidativo del ADNmt
- Mutaciones heteroplásticas y delecciones del ADNmt
- Inhibición directa de la respiración mitocondrial

Otro posible marcador de toxicidad mitocondrial en TAS podría ser el estudio de las enzimas de la cadena respiratoria mitocondrial. Un estudio más reciente[110] parece encontrar relación entre los niveles de las enzimas mitocondriales de los complejos I y IV (CI y CIV) en PBMC y en TAS. Sus niveles están claramente disminuidos tanto en PBMC como en TAS de los pacientes con lipoatrofia.

Sea cual sea el origen de la disfunción mitocondrial, el adipocito es capaz de compensarla[95] a través de diferentes mecanismos como la proliferación mitocondrial, el incremento de captación de la glucosa y la glicolisis. Cuando estos mecanismos resultan insuficientes, aparece el daño celular[111].

3.5.4. Adipocitoquinas. Factor de necrosis tumoral alfa

Ya se ha comentado el papel relevante del tejido adiposo en la secreción de adipocitoquinas (ver figura 8). Éstas tienen un papel importante en la homeostasis energética y metabólica corporal regulando diferentes procesos[112] (tabla 5). Tanto la obesidad como la lipoatrofia se asocian a una disrregulación en la secreción de las adipocitoquinas.

Diferentes adipocitoquinas se han relacionado con la patogénesis de la LD y las alteraciones metabólicas acompañantes. En este sentido, leptina, adiponectina y, en menor grado resistina[113,114,115,116], se han relacionado con dislipemia e insulin-resistencia en pacientes tratados con antiretrovirales que presentan LD. La lipoatrofia se ha correlacionado con niveles bajos de leptina y adiponectina[117,118].

1. Ingesta de comida y balance energético

2. Acción de la Insulina y metabolismo de los hidratos de carbono

3. Metabolismo lipídico

4. Remodelado vascular y angiogénesis

5. Regulación de la tensión arterial y la coagulación

Tabla 5. Procesos regulados por las adipocitoquinas

De entre las adipocitoquinas con actividad inflamatoria, el TNF-α es, probablemente, una de las más destacadas, y motivo de estudio de esta tesis.

TNF-α

El Factor de Necrosis Tumoral alfa se identificó en 1975 como una glicoproteína inducida por una endotoxina que causaba necrosis tumoral en ratones[119]. Desde entonces, se ha implicado en diferentes situaciones inflamatorias, infecciosas y tumorales. La importancia de esta acción, queda reflejada por la eficacia de los tratamientos biológicos con anticuerpos anti-TNF[120]. El gen que lo codifica se encuentra en el brazo corto del cromosoma 6[121].

Producción de TNF-α

El TNF-α se sintetiza en forma de monómero transmembrana de 26 kDa con una proteolisis posterior que libera una proteína soluble, el sTNF-α, que circula en forma de homotrímero facilitando su unión a los receptores. Ambas formas (transmembrana y soluble) presentan actividad biológica[122]. A concentraciones bajas, el TNF-α actúa a nivel paracrino y, a elevadas, pasa al torrente sanguíneo y actúa de forma endocrina[123].

Los macrófagos activados y los linfocitos T son las principales células productoras de TNF-α, aunque mastocitos, células natural killer, células endoteliales, células cardíacas, fibroblastos y osteoclastos pueden secretarlo[120].

El TNF-α no se detecta habitualmente en el sujeto sano pero si en diferentes procesos infecciosos/inflamatorios, tanto en suero como en tejidos y los niveles en suero se correlacionan con la severidad de la infección[124,125].

La producción de TNF-α está, al menos, parcialmente controlada genéticamente[126,127]. Se han descrito diferentes SNP en las posiciones -308 y -238 que se han relacionado con aumento de la expresión del TNF-α[128,129] y 6 diferentes microsatélites (a-f) en la región del promotor del gen del TNF que, también se han relacionado con diferentes niveles de secreción del TNF-α[130].

Receptores de TNF-α

La acción del TNF-α tiene lugar a través de 2 receptores, TNF-α R1 y TNF-α R2. Pertenecen a la superfamilia de los receptores de TNF-α, que incluye hasta 12 miembros, todos ellos con estructura típica transmembrana, pero con diferencias en la unión con su ligando y en su actividad.

TNF-α R1 parece estar más relacionado con los fenómenos de citotoxicidad y apoptosis, mientras que TNF-α R2 interviene más en los procesos metabólicos y de reparación tisular/angiogénesis, aunque no se descarta una acción conjunta en determinadas respuestas[120,131].

Acciones del TNF-α

Las acciones del TNF-α son múltiples. Aplicadas al tejido adiposo, destacan:

1. Acción pro-inflamatoria

Debida tanto su efecto a nivel endotelial, con activación de quimioquinas y moléculas de adhesión (MCP-1, ICAM-1, VCAM-1) que favorecen el reclutamiento de leucocitos a los tejidos como por su producción local por los adipocitos y las células del espacio estromal (macrófagos, preadipocitos…). A nivel adipocitario, el principal estímulo parece ser la apoptosis adipocitaria y el efecto inflamatorio es mediado, principalmente, por el TNF-α R1[122].

El TNF-α inhibe la adipogénesis actuando sobre el PPAR-γ y C/EBPα[132] y sobre genes que regulan el fenotipo de adipocito maduro como aP2[133]. Además, los preadipocitos expresan un marcador temprano de la línea mononuclear-fagocítica, el MOMA-2 (monocyte macrophage-2) y presentan capacidad fagocítica que, en condiciones de inflamación es estimulada. En este ambiente pro-inflamatorio con aumento de los niveles de TNF-α, los preadipocitos pueden convertirse en macrófagos[134,135] perpetuando la inflamación a nivel adipocitario.

3. Metabolismo lipídico y de carbohidratos

TNF-α actua a nivel del metabolismo lipídico y de los carbohidratos favoreciendo la aparición de dislipemia y de resistencia a la insulina / diabetes mellitus clínica. En la tabla 6 se resumen los mecanismos de acción a nivel metabólico.

MECANISMOS DE ACCION	REFERENCIAS
METABOLISMO LIPIDICO	
Inhibición de entrada de AGL	Hauner H 1995[136]
Activación lipólisis	Milles PD 1997[137]
Activación lipogénesis hepática	Kraus RM 1990[138]
METABOLISMO GLUCOSA	
Inhibición del transportador Glut-4	Stephens JM 1992[139]
Inhibición de IRS y de IR	Stephens JM 1997[140]
Inhibición de autofosforilación de IR	Hotamisligil GS 1994[141]

Tabla 6. Mecanismos de acción del TNF-α en el metabolismo lipídico y de los carbohidratos

AGL: Acidos grasos libres. IRS: Insulin Receptor Substrate. IR: insulin Receptor

3.5.5. Efecto del VIH sobre el tejido adiposo

El VIH no infecta directamente a los adipocitos[142]. El estado de inflamación local y sistémica producido por el VIH va a favorecer el paso de los monocitos al tejido adiposo y su activación. Los macrófagos activados secretarán TNF-α y liberarán proteínas virales[143]. De éstas, Nef y Vpr se han implicado en la apoptosis adipocitaria[144, 145]. Recientemente, también se ha demostrado en cultivo de adipocitos[146] que la proteína viral Tat altera la adipogénesis y favorece la liberación de citoquinas proinflamatorias, potenciándose dicho efecto al añadir TNF-α al cultivo celular.

En el tejido adiposo de pacientes infectados por el VIH que no han iniciado TARGA se observa una reducción significativa en la expresión de genes relacionados con la funcionalidad mitocondrial (COII, UCP-2) y el metabolismo lipídico y de los carbohidratos (PPAR-γ, GLUT-4), comparado con el tejido adiposo de pacientes no infectados. Además, existen alteraciones en la expresión de adipocitoquinas (aumento de los niveles de TNF-α y disminución de leptina y adiponectina)[147]

3.5.6. Efecto del TARGA en el tejido adiposo

El TARGA también va a influir en la aparición de LD. Anque la etiopatogenia aún no se conoce, si se cree que es debida, al menos en parte a los efectos de los fármacos antirretrovirales sobre los adipocitos.

Las primeras observaciones sobre la afectación adipocitaria producida por el TARGA datan de finales de los años 90[148, 149]. Usando cultivos celulares (linea celular preadipocitaria 3T3-L1), estos estudios ya mostraban disminución de la expresión de genes pro-adipocitarios y de la concentración de triglicéridos en los adipocitos. Estudios posteriores confirman este efecto antiadipogénico y muestran la capacidad del TARGA de inducir apoptosis "in vitro" e insulinresistencia por inhibición del transportador de Glucosa 4 (Glut-4)[96,150,151]. Los AN, sobretodo d4T y AZT, también afectan al proceso de adipogénesis, aunque en menor medida[152,153].

Ledru et al[154] describieron la desregulación de la homeostasis del TNF-α con acumulación progresiva de células T productoras de TNF-α en pacientes que siguen TARGA como causa posible para la aparición de la LD. A nivel adiposo, estudios "ex vivo" concuerdan en los resultados con los estudios "in vivo", encontrando alteración de la morfología adipocitaria, disminución de los factores proadipogénicos (PPAR-γ, C/EBPα y β, y SREBP-1) y elevación de marcadores inflamatorios y antiadipogénicos como el TNF-α (figura 12)[155,156]
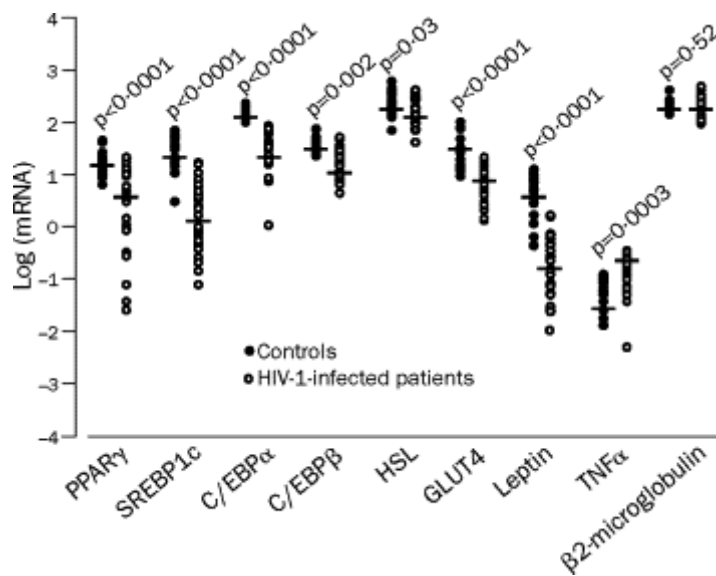
Figura 12. Concentración de mRNA de factores de transcripción y marcadores adipocitarios en tejido adiposo de pacientes VIH con lipoatrofia y controles sanos. Extraído de Bastard et al. Lancet 2002: 359: 1026-31.

3.5.7. Factores relacionados con el huesped

El hecho de que no todos los pacientes infectados por el VIH con TARGA desarrollan LD y/o alteraciones metabólicas ha sugerido el posible papel de una predisposición genética para el desarrollo de la LD[157]. Además de los polimorfismos del TNF-α, han sido varios los genes candidatos estudiados para intentar explicar esta variabilidad. Destacan los siguientes con diferentes resultados (tabla 7)

POLIMORFISMO	AUTOR	RESULTADO
MMP-1	Montes et al 2010[158]	Relación con LD
APM1	Trinca et al 2010[159]	Relación con LD
LEP / LEPR	Trinca et al 2010[159]	No relación con LD
Resistina	Escoté et al 2011[115] Ranade et al 2008[160]	Relación con LD Relación con LD
IL-1β	Asensi et al 2008[161]	Efecto protector contra LD
IL-6	Asensi et al 2008[161] Saumoy et al 2008[162]	No relación con LD No relación con LD
IL-18	Castelar et al 2010[163]	Relación con LD
IFN-γ	Castelar et al 2010[163]	No relación con LD
LMNA	Domingo et al 2002[164] Behrens et al 2003[165]	No relación con LD No relación con LD
B3-adrenergic receptor	Vonkeman et al 2000[166]	Relación con LD
APOC3	Tarr et al 2005[167]	Relación con LD
ESR1 ESR2 gene	Gasparotto et al 2012[168]	Relación con LD en mujeres
PPAR-γ Pro12Ala	Saumoy et al 2009[169]	No relación con LD

Tabla 7: Otros polimorfismos estudiados en relación a la LD

MMP-1 : Metaloproteinasa de la matriz-1. APM1 : Gen de la adiponectina. LEP/LEPR: Gen de la leptina / Gen del receptor de la leptina. IL-1β/6/18: Gen de la Interleuquina 1β/6/18 . IFN-γ: Gen del Interferon gamma. LMNA: Gen de la Lamina. APOC3: Gen de la Apolipoproteína C3. ESR1-2: Gen de los receptores estrogénicos 1 y 2. PPAR-γ pro12Ala: Gen del receptor gamma del proliferador del peroxisoma activado.

En relación al TNF-α, es conocida la desregulación de la homeostasis del sistema del TNF-α en células mononucleares de sangre periférica[154] y las elevadas concentraciones de éste en biopsias de TAS de pacientes con LD[155,170]. Este hecho apoya el posible papel del TNF-α en la LD. Es conocida, además, la asociación, aunque con datos algo contradictorios, del papel de determinados polimorfismos del TNF-α y su relación con niveles aumentados del mismo[35,171].

Se han estudiado diferentes polimorfismos del TNF-α en relación a la aparición de LD en pacientes con infección por VIH. El primer estudio publicado fue realizado por Maher et al[36]. En él, 8 de 35 pacientes con LD eran portadores heterozigotos del polimorfismo -238 G>A del TNF-α mientras que todos los pacientes del grupo control eran portadores del alelo común. Estos datos sugerían una posible asociación entre esta variante genética y el desarrollo de LD.

Un segundo estudio publicado, de la cohorte australiana[172], mostró una relación entre ser portador del polimorfismo -238 G>A y la rapidez de desarrollar lipodistrofia en el tiempo.

Otros estudios, sin embargo, no encuentran relación entre los polimorfismos del TNF-α y la aparición de LD en pacientes infectados por el VIH[161,167].

Un estudio italiano reciente que incluía a 151 pacientes[173] mostró una relación independiente entre ser portador del polimorfismo -308 GG y el riesgo aumentado de presentar acúmulo de grasa a nivel troncal, además de no encontrar relación entre los polimorfismos de TNF -238 y la presencia de lipoatrofia.

Un estudio brasileño[130] encontró asociación entre ser portador del alelo -308G con la susceptibilidad de presentar LD y el microsatélite TNF-α5 como factor protector contra la LD.

3.5.8. Teoría inflamatorio. Visión global

Con todos los datos expuestos anteriormente, se puede afirmar que la inflamación del tejido adiposo es uno de los mecanismos que contribuyen a la aparición de LD[111].

Una visión global y cronológica del fenómeno inflamatorio en la etiopatogenía de la LD en los pacientes infectados por VIH podría ser la siguiente:

1. La propia infección por VIH produce un sustrato inflamatorio con aumento de los niveles de TNF-α a nivel adipocitario mediado por proteínas virales como Nef y Vpr.

2. Sobre ese sustrato, incide el uso prolongado del TARGA, sobretodo la combinación de AN e IP, produciendo:

   - Disregulación de la producción de TNF-α
   - Disminución de la diferenciación adipocitaria y aumento de la apoptosis
   - Paso de pre-adipocito a macrófago
   - Secreción aumentada de citoquinas pro-inflamatorias y factores quimiotácticos

3. Un contexto de posible predisposición genética, todavía por definir.

Este proceso provocaría una mayor atracción de macrófagos y perpetuación del fenómeno inflamatorio en TAS, apareciendo el fenotipo característico de la LD y las alteraciones metabólicas asociadas. La figura 13 resume estos conceptos[174].
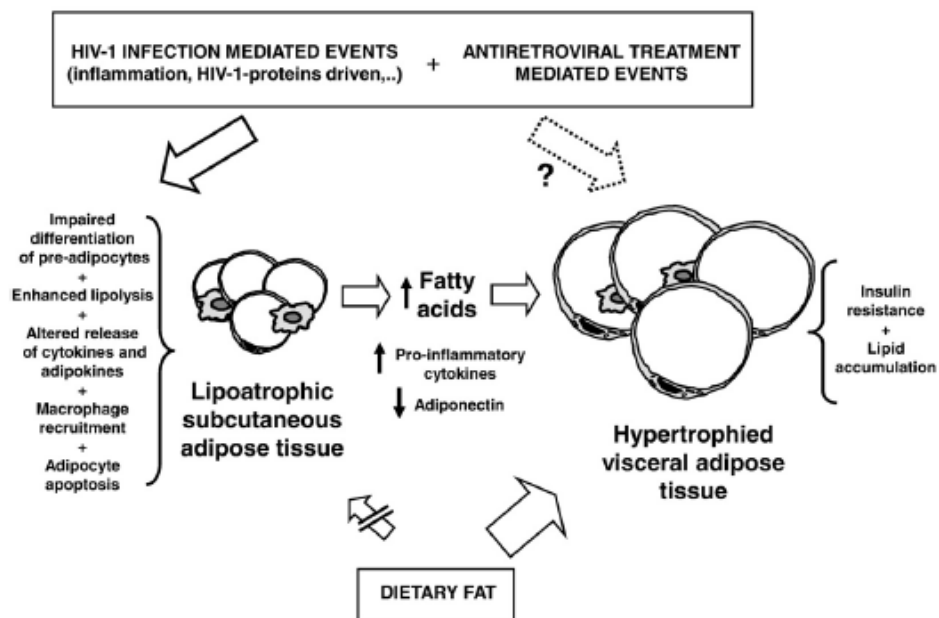
Figura 13. Impacto de la infección por VIH y el TARGA sobre el tejido adiposo. Extraído de Villarroya F et al. Biochimic Biophys Acta 2010; 1801 (3): 392-9

3.6. Tratamiento

No existe, en la actualidad, un tratamiento adecuado para la LD. Las primeras intervenciones fueron el cambio de IP por NAN. Este cambio mejoró el perfil lipídico pero no hubo regresión de los cambios en tejido adiposo[175,176,177].

Al descubrirse la disfunción mitocondrial por AN, se realizaron varios estudios de sustitución de AN tipo timidínico (AZT y d4T) por otros menos lesivos a nivel mitocondrial (TDF y ABC). Ésta es la única intervención efectiva en mejorar la lipoatrofia, demostrada en diferentes estudios de manera independiente[178].

Los estudios TARHEEL[179], MITOX[180] y RAVE[181] encuentran mejoría en la lipoatrofia subcutanea tras el cambio de los AN. Así mismo, el inicio del TARGA con AN menos lesivos muestra una disminución de la incidencia de LD[73,182,183,184].

Otro abordaje terapéutico en relación con la lipoatrofia es el tratamiento quirúrgico de la misma mediante implantes. Las técnicas disponibles son el trasplante autólogo de grasa, inyecciones de material biodegradable (ácido poliláctico) y material no biodegradable (polimetilmetacrilato).[185] La elección de la técnica va a depender de las características de cada paciente. A veces, se requiere de reintervenciones y los resultados, en general, son satisfactorios para el paciente, mejorando claramente su percepción y calidad de vida.

En relación con la lipohipertrofia, se han utilizado agentes insulinosensibilizantes como las tiazolidinedionas (Troglitazona, Roxiglitazona) y Metformina. Los limitados resultados obtenidos y su toxicidad hacen que su uso no esté indicado[186,187,188,189,190].

La tesamorelina, análogo sintético del factor liberador de la hormona de crecimiento (GHRF), es el único fármaco aprobado por la FDA, actualmente, para el tratamiento de la lipohipertrofia[191] (Traynor K 2010). Dos estudios multicéntricos demostraron un descenso de la grasa visceral de hasta un 18% a las 52 semanas de tratamiento con aceptable tolerancia clínica. Al retirar el fármaco, el efecto revertía[192].

B. JUSTIFICACION DE LA PRESENTE TESIS

La presente tesis estudia la implicación del sistema del TNF-α en dos aspectos de la infección por VIH. El primero de ellos en relación con la inmunopatogenia de la infección y el segundo con la aparición del síndrome de lipodistrofia en pacientes que reciben tratamiento antirretroviral.

En ambos casos, las citoquinas juegan un papel importante, regulando la replicación viral en la inmunopatogenia y los fenómenos inflamatorios que suceden en la LD.

La importancia del sistema TNF-α hace que sea un candidato ideal para estudiar su participación en ambos aspectos de la infección.

Los resultados de la presente tesis se exponen en base a tres artículos publicados en diferentes revistas médicas de ámbito internacional. Posteriormente, se realiza una discusión de los resultados obtenidos y, finalmente, se exponen las conclusiones alcanzadas a partir de dichos resultados

C. HIPOTESIS Y OBJETIVOS

1. HIPOTESIS

1.1. TNF-a y lipodistrofia

El propio VIH, la medicación antirretroviral y la susceptibilidad genética del huésped parecen tener un papel importante en la etiopatogenia de la LD que se observa en pacientes infectados por el VIH tratados con fármacos antiretrovirales.

Dentro de esta etiología multifactorial, la existencia de un ambiente proinflamatorio en estos pacientes hace pensar que diferentes citoquinas proinflamatorias van a jugar un papel destacado y, de éstas, la más importante es el Factor de Necrosis Tumoral alfa (TNF-a).

Pensamos pues que el TNF-a puede estar implicado en la etiopatogenia de la LD asociada al VIH y al TARGA.

1.2. TNF-a e historia natural de la infección por el VIH

Algunas investigaciones sugieren que la infección por el VIH es una enfermedad mediada por citoquinas, entre ellas el TNF-a. La producción de TNF-a está mediada, al menos en parte, genéticamente.

La información que existe en relación al papel de las variantes genéticas de TNF-a y su impacto en la historia natural de la infección por el VIH es discordante

Pensamos que los polimorfismos en el gen que codifica para TNF-a podrían estar asociados a diferentes patrones evolutivos de la infección por el VIH.

2. OBJETIVOS

2.1. TNF-α Y LIPODISTROFIA

Objetivo primario

1. Estudiar la posible relación entre los niveles de TNF-α y la presencia de apoptosis en el tejido adiposo subcutáneo.

2. Estudiar la posible asociación entre los polimorfismos del TNF-α y la aparición de lipodistrofia.

Objetivos secundarios

1. Estudiar el papel del TNF-α y del TARGA en la apoptosis "*in vitro*" utilizando cultivos celulares de adipocitos.

2.2 TNF-α e historia natural de la infección por VIH

Objetivo primario

1. Evaluar la relación entre los polimorfismos de TNF-α con el riesgo de infección por VIH y la diferente progresión de la enfermedad.

Objetivo secundario

1. Evaluar la relación entre CCR5Δ32 con el riesgo de infección por VIH y la diferente progresión de la enfermedad.

D. PUBLICACIONES

ESTUDIO 1

ORIGINAL ARTICLE

Tumour necrosis factor alpha in fat redistribution syndromes associated with combination antiretroviral therapy in HIV-1 infected patients: potential role in subcutaneous adipocyte apoptosis

P. Domingo, F. Vidal, J.C. Domingo*, S. Veloso*, M.A. Sambeat, F. Torres, J.J. Sirvent. J. Vendrell, X. Matias-Guiu and C. Richart, on behalf of the HIV-FRS Study Group.

Factor de impacto (2011): 2,643

Tumour necrosis factor alpha in fat redistribution syndromes associated with combination antiretroviral therapy in HIV-1-infected patients: potential role in subcutaneous adipocyte apoptosis

P. Domingo[*], **F. Vidal**[†], **J. C. Domingo**[‡], **S. Veloso**[†], **M. A. Sambeat**[*], **F. Torres**[§], **J. J. Sirvent**[†], **J. Vendrell**[†], **X. Matias-Guiu**[¶] and **C. Richart**[†], on behalf of the HIV-FRS Study Group[**]

[*]Autonomous University of Barcelona, Barcelona, [†]Rovira i Virgili University, Tarragona, [‡]University of Barcelona, Barcelona, [§]Autonomous University of Barcelona, Barcelona; [¶]University of Lleida, Lleida, Spain

Abstract

Background The pathogenesis of fat redistribution syndromes (FRS) observed in the setting of highly active antiretroviral therapy (HAART) for the treatment of HIV-1-infection remains elusive. A dysregulation of the tumour necrosis factor alpha (TNF-α) system occurs in HIV-infected patients with FRS.

Materials and methods The study looked at both the *in vivo* and *in vitro* relationship between TNF-α and the degree of subcutaneous adipocyte apoptosis in 60 HIV-1-infected patients on HAART with FRS, another 60 HIV-1-infected patients on HAART without FRS and 60 uninfected control patients. Apoptosis was assessed by the terminal deoxynucleotidyl transferase dUTP (deoxyuridine 5′-triphosphate)-digoxigenin Nick End Labelling (TUNEL) method. Soluble receptors of TNF-α were determined by the sandwich enzyme immunoassay technique. The *in vitro* viability was assessed by staining with 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) and apoptosis by TUNEL.

Results HIV-1-infected patients with FRS had significantly higher degrees of subcutaneous adipocyte apoptosis than those without FRS ($P = 0.0001$) and uninfected controls ($P < 0.0001$). There was a statistically significant association between serum levels of soluble TNF-α receptors #1 and #2 and the degree of subcutaneous adipocyte apoptosis in patients with and without FRS ($P < 0.0001$ for both receptors). *In vitro*, the addition of TNF-α (10 ng mL^{-1}) to an adipocyte culture embedded with indinavir, either alone or in clinically relevant combinations with stavudine (d4T) and lamivudine (3TC), significantly decreased adipocyte viability ($P = 0.0001$) and increased adipocyte apoptosis ($P < 0.0001$) with respect to that observed with the addition of antiretrovirals alone.

Conclusions TNF-α plays a significant role in subcutaneous adipocyte apoptosis, which occurs in the setting of FRS in HIV-1-infected patients on highly active antiretroviral therapy.

Keywords Highly active antiretroviral therapy, HIV-1 infected, lipodystrophy, subcutaneous adipocyte apoptosis, TNF-α receptors, TNF-α.

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Introduction

Subcutaneous adipocyte apoptosis has been described as occurring in lipoatrophic areas of human immunodeficiency virus (HIV-1)-infected patients with fat redistribution syndromes (FRS) [1,2]. However, the factors triggering the apoptotic sequence are far from being known, although TNF-α could be a strong candidate as it is a potent inducer of adipocyte apoptosis *in vitro* and *in vivo* [3–5]. It has also been described that dysregulation of TNF-α metabolism occurs in HIV-1-infected patients on combination antiretroviral therapy who develop FRS [6]. Regulation of TNF-α signalling is complex and its action appears to occur via an autocrine-paracrine mechanism in skeletal and adipose tissues. The TNF-α signalling occurs through at least two cell-surface receptors, TNFR1 and TNFR2, that are present in virtually all cells of higher mammals, including adipocytes. Additionally, both protease inhibitors and nucleoside reverse transcriptase inhibitors, especially thymidine analogues, have demonstrated *in vitro* their potential to cause adipocyte apoptosis [7]. *In vivo*, the degree of subcutaneous adipocyte apoptosis may, or may not, be modified by switching potentially implicated drugs [2,8].

The aim of this work was to investigate the possibility that TNF-α, leptin or IL-6 could be related to the ongoing adipocyte apoptosis that takes place in the subcutaneous fat of lipoatrophic areas of HIV-infected patients on HAART, suggesting a possible mechanism operating in FRS. The work was designed as a two-phase study; the first phase to investigate the *in vivo* relationship between TNF-α system activation, measured by the soluble fraction of TNFR1 and R2 and adipocyte apoptosis in patients with and without FRS treated with HAART, and the second phase was to assess the positive findings of the first phase on an experimental *in vitro* model.

Materials and methods

Subjects

Patients were prospectively and consecutively included in a study of sequential analysis of fat changes, provided that they had HIV-1 infection, were receiving HAART and had, or had not, the clinical characteristics consistent with FRS associated with antiretroviral therapy. The FRS was defined by severe fat wasting from the face, buttocks, limbs and upper trunk with, or without, central adiposity, buffalo hump or breast hypertrophy in women according to previously defined criteria [9]. All the patients with FRS had marked self-reported body shape changes that were ascertained by the examining physician. Central obesity was defined by a waist-hip ratio > 0·95 in men and > 0·85 in women [10]. Exclusion criteria were the presence of wasting syndrome defined on the basis of the CDC criteria [11] and the presence of concomitant opportunistic infections, or other inflammatory conditions, when the levels of soluble TNF receptors (sTNFRs) were measured. Concomitant

serum determination of C-reactive protein (CRP) levels was also performed to eliminate infections or other inflammatory conditions. Alcohol abuse (defined as daily alcohol consumption > 40 g) and the use of drugs with metabolic effects or with known effects on fat distribution or cytokine metabolism (i.e. anabolic hormones, corticosteroids, exogenous cytokines) were also exclusion criteria. Sixty healthy subjects, matched by age and sex to the HIV-infected patients with FRS, were used as the controls with regard to cytokine, and 24 had subcutaneous fat biopsies for apoptosis measurements. There were no statistically significant differences in clinical and biochemical parameters between the controls biopsied and those who were not. Ethical Committees of both participating hospitals approved the study protocol. Informed consent was obtained from all subjects involved in the study.

Laboratory methods

Plasma HIV-1 RNA concentrations were determined with the Roche Amplicor HIV-1 Monitor assay (Roche Diagnostic Systems, Basel, Switzerland) which has a lowest detection limit of 20 copies μL^{-1} [12]. Results below the assay limit of detection were assigned a value of $1·28 \log_{10}$ copies mL^{-1} plasma. Changes in CD4 and CD8 lymphocytes were the differences between pre-HAART and study measurements. The CRP was measured by the turbidimetic method. Blood samples were collected and glucose and lipid measurements were performed as previously described [13]. Serum leptin concentration was measured by radio-immunoassay (RIA) according to commercial kit instructions, Human Leptin RIA kit, (Linco Research Inc., St. Charles, MO), where the lower limit of detection was $0·5 ng mL^{-1}$. Coefficients of variation intra- and interassay were < 7% and < 8%, respectively. Serum insulin level was measured by RIA, Human Insulin Specific RIA Kit, (Linco Research, St. Louis, MO). The sensitivity of the assay was $2 \mu IU mL^{-1}$. Coefficients of variation intra- and interassay were < 1% and 0·67–7·43%, respectively. Insulin resistance was calculated according to the homeostasis model assessment for insulin resistance (HOMA-r) method from fasting glucose and insulin concentrations, according to the formula: insulin ($\mu IU mL^{-1}$) × glucose (mmol L^{-1})/22·5 [14].

Cytokine measurements

Soluble receptors of TNF-α (sTNFR1 and sTNFR2) were determined by the sandwich enzyme immunoassay technique according to commercial kit instructions (EASIA, Biosource Europe, Fleunes, Belgium). The lower limit of detection for sTNFR1 and sTNFR2 was $0·1 ng mL^{-1}$. Coefficients of variation intra- and interassay were < 7% and < 9%, respectively. There was no cross-reaction between them and with TNF-α. The IL-6 was measured by immunoassay kit, Human IL-6 US Ultrasensitive (R&D Systems, Abingdon, UK). Coefficients of variation intra- and interassay were < 7% and < 9%, respectively.

Pathological methods and assay for apoptosis

A biopsy of subcutaneous fat was performed on the antero-lateral aspect of the right leg and the samples were submitted for standard pathologic study together with a careful search for adipocyte apoptosis, previously described elsewhere [1,2]. Apoptosis was scored semiquantitatively: (1) negative, when there were no positive nuclei; (2) focally positive, one or two positive nuclei in 20 high-power fields; (3) moderate positively (three to five positive nuclei in 20 high-power fields); and (4) diffusely positive (more than five nuclei in 20 high-power fields) [1,2]. Two pathologists evaluated each sample in a blinded manner, and in discordant interpretations a consensus report was invoked.

Culture, differentiation and treatment of 3T3-L1 cells

Preadipocytes, 3T3-L1, were obtained from the American Tissue Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% newborn calf serum and were differentiated as described previously [15]. After 2–3 days from confluence, differentiation was induced by treatment with a mixture of insulin ($0{\cdot}1$ U mL^{-1}), isobutylmethylxanthine ($0{\cdot}5$ mM) and dexamethasone (1 mM) in DMEM medium. After 48 h in the differentiation medium it was changed into post-differentiation medium (without dexamethasone or isobutylmethylxanthine). At day 7 in the post-differentiation medium, 90% of the cells had differentiated. Thereafter, this medium was changed every 2 days. All experiments were performed on day 9 or 10 subsequent to the initiation of adipocyte differentiation. At this point, at least 90% of 3T3-L1 cells showed accumulation of fat droplets.

Powdered forms of indinavir, d4T and 3TC were provided by Merck Sharp & Dohme Laboratories (New Brunswick, NJ, USA), Bristol-Myers-Squibb Laboratories (West Point, PA, USA) and Glaxo-Welcome Laboratories (Hertfordshire, UK), respectively. Stock solution (100-mM concentration) of indinavir was prepared in dimethyl sulfoxide (DMSO) and d4T and 3TC were made in phosphate-buffered saline (PBS). In culture medium, the DMSO final concentration was less than $0{\cdot}1\%$ (V/V) and a control was cultured with the same concentration. At 24 h after the cells were plated, fresh medium containing the drugs at 20 μM of individual concentration was added and the cells were maintained in the presence of the drugs for 3 days. Where indicated, recombinant murine TNF-α, 2×10^6 U mg^{-1} (Sigma, St. Louis, MO) dissolved in PBS containing $0{\cdot}1\%$ fatty acid-free and growth factor-depleted bovine serum albumin (Sigma, St. Louis, MO) was diluted in medium to obtain a final concentration of 10 ng mL^{-1}.

Assessment of cell viability and apoptosis

Viability was assessed by staining with 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT). Briefly,

cells were plated at 2×10^4/well for adipocytes and incubated for 72 h in medium without or with drugs and/or TNF-α. Cells were incubated in 1 mg mL^{-1} MTT for 3 h at 37 °C. Supernatants were discarded and absorbance of cells dissolved in isopropyl alcohol was measured at 570 nm on an ELISA reader. Cell survival was calculated as the mean of triplicate OD values of cells incubated and divided by the mean of triplicate OD values of cells incubated in control medium and expressed as a percentage. In parallel, DNA fragmentation associated with apoptotic cell death was studied by TUNEL (TdT-mediated dUTP nick-end labelling) assay. Adipocyte cells (1×10^6 mL^{-1}) were processed according to the ApoAlert DNA fragmentation kit protocol supplied by the manufacturer (Clontech Laboratories Inc., Palo Alto, CA). The FITC-labelled positive cells were identified by flow cytometry. A total of 10 000 cells/sample were analyzed and FACS analysis was performed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm Argon laser. The CellQUEST software (Becton Dickinson) was used for data analysis.

Statistical analysis

Although the primary objective was to test the effect of the TNF-α on adipocyte apoptosis, there was no formal working hypothesis because of an absence of information on the expected magnitude of that effect; therefore, the sample size was not predetermined on statistical computations. However, the sample size appeared to be reasonable according to the standard sample sizes for these types of studies [22,31]. A non-parametric ANOVA, by means of a rank transformation on the dependent variable, was used to analyze quantitative and ordinal variables; post-hoc pair-wise comparisons were adjusted by the Bonferroni method [16]. When applicable, a trend-test was also performed. To ascertain the viability assays and the apoptosis experiments, both the antiretroviral treatment, the TNF-α expositions and also their interaction were evaluated in the model. Spearman's rho statistic was used to test linear relationship between variables. The analysis was performed using SAS ver $8{\cdot}2$ software (SAS Institute, Cary, NC, USA) and the level of significance was established at the $0{\cdot}05$ level (two-sided).

Results

Population studied

During the period January 2002 to September 2003, a biopsy sample size large enough to be analyzed was obtained from 120 patients and 24 control participants. Sixty of the patients who were HIV infected on HAART had FRS (FRS+) and the remaining 60 patients were on HAART without FRS (FRS−). Population demographics, treatment, immunological and virological data and

Table 1 Demographics and antiretroviral treatment data of patients and controls

	HAART, FRS+ (*n* = 60)	HAART, FRS– (*n* = 60)	Controls (*n* = 60)	*P* value
Age (years)	42 (36·0–50·5)	39·5 (35·0–47·0)	42 (36·0–50·5)	0·3381
Sex (% men)	55	60	55	0·81
BMI	23·5 (21·9–25·7)	23·0 (21·1–25·8)	26·7 (24·0–30·6)	< 0·0001
WHR	0·94 (0·89–0·98)	0·89 (0·85–0·92)	0·85 (0·85–0·93)	< 0·0001
Duration of HIV infection (m)	97·5 (67–130)	93·5 (69–139·5)	–	0·8347
Duration of HAART (m)	60 (49–67)	60·5 (47–80·5)	–	0·5605
Cumulative time on NRTIs (m)	124 (104–157·5)	106 (85–156)	–	0·1959
Cumulative time on d4T (m)	47·5 (26·5–54·0)	6 (0–31·0)	–	< 0·0001
Cumulative time on AZT (m)	12·5 (2·0–37·0)	41 (20·0–59·0)	–	< 0·0001
Cumulative time on ddC (m)	11 (5·0–17·2)	6·5 (1·0–27·0)	–	0·46
Cumulative time on ddI (m)	23 (9·2–36·5)	12 (6·2–32·7)	–	0·42
Cumulative time on 3TC (m)	47 (29·2–56·0)	43 (28·0–58·0)	–	0·88
Cumulative time on ABC (m)	10 (7·2–13·5)	31 (22·5–39·2)	–	0·0014
Cumulative time on TDF (m)	2·5 (2·0–3·0)	4·5 (3·0–7·0)	–	0·09
Cumulative time on NVP (m)	19·5 (12·5–33·5)	14·5 (5·0–38·0)	–	0·49
Cumulative time on EFV (m)	12 (8·7–15·7)	34 (14·2–42·5)	–	0·0035
Cumulative time on IDV (m)	19 (7·0–32·0)	22 (11·5–34·0)	–	0·37
Cumulative time on SQV (m)	14 (10·7–30·0)	36 (13·2–51·2)	–	0·04
Cumulative time on RTV (m)	6 (2·0–20·0)	12 (8·0–31·2)	–	0·09
Cumulative time on NFV (m)	22·5 (12·0–38·0)	27 (9·5–34·0)	–	0·092
Cumulative time on APV (m)	3 (–)	31 (–)	–	0·31
Cumulative time on LPV/r (m)	12 (8·0–20·0)	20 (13·0–32·5)	–	0·36
Cumulative time on PIs (m)	34·5 (23·0–53·0)	38 (20–64)	–	0·5626
Cumulative time on NNRTIs (m)	13·5 (1·5–24·0)	11·5 (0–38)	–	0·8885

All *P* values are significance tests, values are expressed as median (interquartile range).

ABC, abacavir; APV, amprenavir; AZT, zidovudine; BMI, body mass index; ddC, zalcitabine; ddI, didanosine; d4T, stavudine; EFV, efavirenz; FRS, fat redistribution syndromes; HAART, highly active antiretroviral therapy; IDV, indinavir; LPV/r, lopinavir/ritonavir; m, months; NFV, nelfinavir; NNRTIs, non-nucleoside reverse transcriptase inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NVP, nevirapine; Pis, protease inhibitors; RTV, ritonavir; SQV, saquinavir; TDF, tenofovir; WHR, waist-to-hip ratio; 3TC, lamivudine.

cytokine measurements are shown in Tables 1 and 2. The majority of the HIV-1-infected patients (FRS+ , 56%; FRS–, 46·7%) had acquired HIV-1-infection through heterosexual transmission (*P* = 0·75). Most of the patients had been exposed to the three classes of antiretrovirals, and the cumulated exposure for each antiretroviral class is shown in Table 1. The most frequently used combinations were d4T+3TC+indinavir (17 patients, 14·2%) and d4T+3TC+nelfinavir (10 patients, 8·35%). Sixty patients (50%) were taking a protease inhibitor-based regime, whereas 48 (40%) were taking a non-nucleoside reverse transcriptase inhibitor-based regime. From a morphologic aspect, 14 patients (23·3%) of the FRS group had isolated lipoatrophy and 46 (76·7%) had a mixed syndrome [15].

Cytokine levels and metabolic parameters

The median serum levels of cytokines are shown in Table 2. There were statistically significant differences within the three studied groups with respect to sTNFR1 (*P* < 0·0001, trend-test *P* = 0·0273), sTNFR2 (*P* < 0·0001, trend-test *P* = 0·0006), interleukin-6 (*P* = 0·0115, trend-test *P* = 0·0056) and leptin (*P* < 0·0001, trend-test *P* = 0·007) (Table 2).

Serum levels of sTNFR1 correlated with sTNFR2 (*r* = 0·42, *P* < 0·0001), triglycerides (*r* = 0·22, *P* = 0·0037), HDL (*r* = –0·21, *P* = 0·005) and HOMA-r (*r* = 0·19, *P* = 0·04). Serum levels of sTNFR2 correlated with triglycerides (*r* = 0·34, *P* < 0·0001), negatively with HDL cholesterol (*r* = –0·29, *P* < 0·0001) and with HOMA-r (*r* = 0·36, *P* < 0·0001). Serum leptin levels correlated with BMI (*r* = 0·26, *P* = 0·0006). No association was found between sTNFR levels and individual antiretroviral drug exposure.

Subcutaneous adipocyte apoptosis and cytokine levels

Fifty-two patients' specimens (43·3%) were negative for the presence of apoptosis, 35 (29·2%) showed focally positive apoptotic cells, 20 (16·7%) had moderate apoptotic changes and the remaining 13 biopsies (10·8%) demonstrated diffuse positivity, whereas all the control specimens were negative (Table 3). The degree of subcutaneous adipocyte apoptosis was not statistically associated with age, duration of HIV infection, anthropometric measures, decrease in viral load, change in CD4 or CD8 cells, total cholesterol or LDL cholesterol. However, a longer time on HAART was associated with the degree of subcutaneous adipocyte apoptosis (*P* = 0·0385) and with HOMA-r (*P* = 0·00112),

Table 2 Metabolic parameters and cytokine levels of patients and controls

	HAART, FRS+ ($n = 60$)	HAART, FRS– ($n = 60$)	Controls ($n = 60$)	P value[*]
CRP (mg mL^{-1})	3·3 (3·1–3·8)	3·3 (3·0–3·7)	3·4 (3·2–4·0)	0·07
Glucose (mmol L^{-1})	5·1 (4·7–5·7)	4·8 (4·5–5·5)	4·8 (4·5–5·5)	< 0·0001
Triglycerides (mmol L^{-1})	2·2 (1·6–3·8)	1·8 (1·2–2·4)	0·9 (0·6–1·4)	< 0·0001
Total cholesterol (mmol L^{-1})	5·8 (4·4–6·7)	5·2 (4·4–5·7)	5·0 (4·4–5·7)	0·0150
HDL cholesterol (mmol L^{-1})	1·0 (0·9–1·3)	1·2 (1·0–1·4)	1·4 (1·2–1·6)	< 0·0001
LDL cholesterol (mmol L^{-1})	3·8 (2·8–4·4)	3·1 (2·4–3·5)	3·3 (2·5–3·8)	0·0085
HOMA-r	3·6 (1·9–6·5)	2·1 (1·2–4·1)	1·3 (0·9–2·0)	< 0·0001
CD4 count (cells cμ^{-1} mm)	580 (427–875)	648 (510–851)	–	0·33
CD4 change (cells cμ^{-1} mm)	290 (133–548)	419 (284–581)	–	0·1269
CD8 change (cells cμ^{-1} mm)	433 (40–718)	96 (−252–426)	–	0·0002
HIV-1 RNA (log$_{10}$ copies mL^{-1})	1·28 (1·28–1·79)	1·28 (1·28–1·31)	–	0·29
Decrease in viral load (log$_{10}$)	3·1 (1·9–4·1)	3·5 (3·0–4·3)	–	0·0451
sTNFR1 (ng mL^{-1})	1·9 (1·5–2·8)	1·7 (1·3–2·1)	1·5 (1·2–1·8)	< 0·0001 0·0273[*]
sTNFR2 (ng mL^{-1})	5·4 (4·2–7·6)	4·4 (3·0–5·8)	3·3 (2·8–4·1)	< 0·0001 0·0006[*]
Leptin (ng mL^{-1})	3·8 (2·1–7·5)	5·3 (2·7–9·1)	7·6 (4·7–17·7)	< 0·0001 0·0070[*]
Interleukin-6 (ng mL^{-1})	1·8 (1·2–2·6)	1·8 (0·9–2·7)	3·0 (2·0–3·0)	0·0115 0·0056[*]

All P values are significance tests except those marked ([*]) which correspond to trend-tests; values are expressed as median (interquartile range).

CRP, C–reactive protein; FRS, fat redistribution syndromes; mmol L^{-1}, millimols per litre; HDL, high density lipoprotein; LDL, low density lipoprotein; HOMA-r, homeostasis model assessment for insulin resistance; sTNFR, soluble tumour necrosis factor receptor; ng mL^{-1}, nanograms per millilitre.

Statistically significant Bonferroni adjusted pair-wise comparisons: triglycerides (FRS+ vs. controls, $P < 0·0001$; FRS– vs. controls, $P < 0·0001$; FRS+ vs. FRS–, $P = 0·0085$); total cholesterol (FRS+ vs. controls, $P = 0·0206$; FRS– vs. controls, $P = 1$; FRS+ vs. FRS–, $P = 0·0727$), HDL cholesterol (FRS+ vs. controls, $P < 0·0001$; FRS– vs. controls, $P = 0·0445$; FRS+ vs. FRS–, $P = 0·0238$), LDL cholesterol (FRS+ vs. controls, $P = 0·0961$; FRS– vs. controls, $P = 1$; FRS+ vs. FRS–, $P = 0·0076$); HOMA-r (FRS+ vs. controls, $P < 0·0001$; FRS– vs. controls, $P = 0·0091$; FRS+ vs. FRS–, $P = 0·0139$); sTNFR1 (FRS+ vs. controls, $P < 0·0001$; FRS– vs. controls, $P = 0·0659$; FRS+ vs. FRS–, $P = 0·0878$); sTNFR2 (FRS+ vs. control, $P < 0·0001$; FRS– vs. control, $P = 0·0005$; FRS+ vs. FRS–, $P = 0·0029$); Interleukin-6 (FRS+ vs. controls, $P = 0·0438$; FRS– vs. control, $P = 0·0243$; FRS+ vs. FRS–, $P = 1$); Leptin (FRS+ vs. control, $P < 0·0001$; FRS– vs. control, $P = 0·0219$; FRS+ vs. FRS–, $P = 0·1774$).

Table 3 Degree of subcutaneous adipocyte apoptosis and the presence or absence of FRS in HIV-1-infected patients on combination antiretroviral therapy and noninfected controls

	HIV-1-infected patients		
Degree of apoptosis	HAART, FRS+ ($n = 60$)	HAART, FRS– ($n = 60$)	Controls ($n = 24$)
Negative (%)	17 (28·3)	35 (58·3)	24 (100)
Focal (%)	20 (33·3)	15 (25·0)	0 (0)
Moderate (%)	10 (16·7)	10 (16·7)	0 (0)
Diffused (%)	13 (21·7)	0 (0)	0 (0)

FRS, fat redistribution syndromes; global significance test $P < 0·0001$; pair-wise Bonferroni adjusted comparisons (FRS vs. controls, $P < 0·0001$; no FRS vs. controls, $P = 0·0021$; FRS vs. no FRS $P < 0·0001$).

whereas cumulative time of NRTIs, cumulative time on protease inhibitors or other antiretroviral drugs were not. No association was found between apoptosis and individual drug exposure, including that of d4T. The degree of subcutaneous apoptosis was different between groups (test of global significance $P < 0·0001$) with all pair-wise Bonferroni-adjusted comparisons being statistically significant (Table 3). Serum levels of both sTNFR1 and sTNRF2 were significantly associated with the degree of subcutaneous adipocyte apoptosis ($P < 0·0001$, for both) (Figs 1 and 2). Post-hoc between-pattern comparisons showed that statistical differences for sTNFR1 were established on the basis of differences between patients with diffuse apoptosis and those exhibiting negative, focal or moderate degrees of apoptosis (Fig. 1). Post-hoc between-pattern comparisons also showed that statistical differences for sTNFR2 were established on the basis of differences between patients with diffuse apoptosis and those exhibiting negative, focal or moderate degrees of apoptosis, but also between patients with negative and those with focal or moderate findings (Fig. 2). When studying sTNFR1 in FRS+ and FRS– patients by means of a two-way non-parametric ANOVA, the degree of apoptosis was clearly associated ($P < 0·0001$) but the subject group was not statistically significant ($P = 0·7583$)
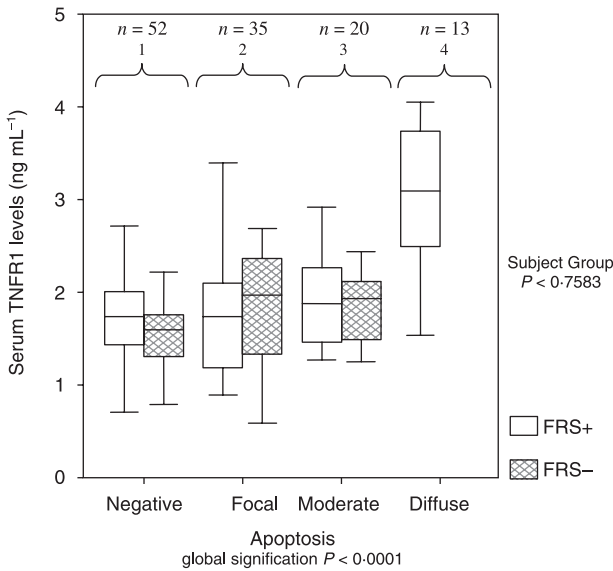
Figure 1 Serum levels of sTNFR1 in HIV-1-infected patients with combination antiretroviral therapy, with or without fat redistribution with respect to the degree of subcutaneous adipocyte apoptosis. Global statistical signification: $P < 0.0001$, ranked-ANOVA; significant Bonferroni-adjusted post-hoc comparisons: negative (1) vs. diffuse (4), $P = 0.0001$; focal (2) vs. diffuse (4), $P = 0.0013$; moderate (3) vs. diffuse (4), $P = 0.0360$; and trend tests $P < 0.0001$.
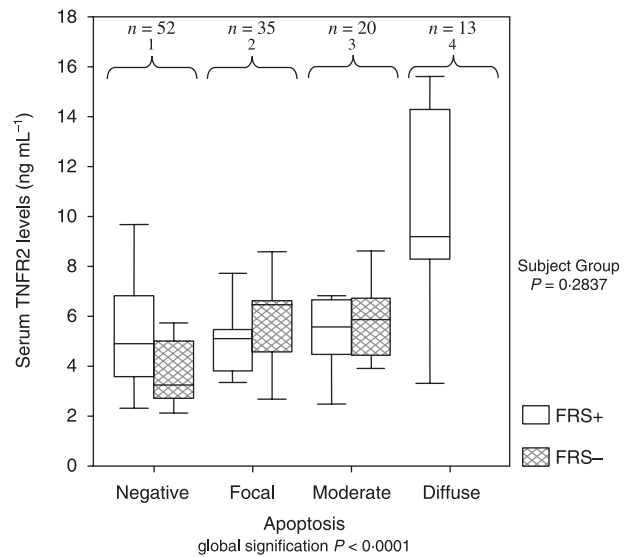
Figure 2 Serum levels of sTNFR2 in HIV-1-infected patients with combination antiretroviral therapy, with or without fat redistribution with respect to the degree of subcutaneous adipocyte apoptosis. Global statistical signification: $P < 0.0001$, ranked-ANOVA; significant Bonferroni-adjusted post-hoc comparisons: negative (1) vs. focal (2), $P = 0.0106$; negative (1) vs. moderate (3), $P = 0.0032$; negative (1) vs. diffuse (4), $P = 0.0001$; and focal (2) vs. diffuse (4), $P = 0.0268$; trend tests $P < 0.0001$.

(Fig. 1). The same conclusions arise from the analysis of sTNFR2 where the degree of apoptosis was also associated ($P < 0.0001$) and the subject group was not significant ($P = 0.2837$) (Fig. 2). In addition, the trend-test was also significant ($P < 0.0001$ for TNFR1 and TNFR2). This indicated that there was a relationship between the degree of apoptosis and both TNFR1 and TNFR2 independently from the kind of patient (FRS+ vs. FRS–) and that this relationship followed a lineal trend. Conversely, neither leptin nor interleukin-6 serum levels showed any statistically significant result when the same approach was performed.

Experimental adipocyte viability, adipocyte apoptosis, and TNF-α

When adipocytes were cultured, in accordance with previous studies [5], a decrease (10%) in cell survival, measured with the use of the vital stain MTT, was observed (Fig. 3). To evaluate the interference of antiviral drugs (indinavir, NRTIs or combination) in the viability of adipocytes, 3T3-L1 cells were maintained in the presence of antiretroviral drugs at concentrations similar to those observed in plasma of treated patients in the absence, or presence, of TNF-α. Both the drug treatment and the TNF-α factors were statistically associated ($P < 0.0001$ for both) in the two–way nonparametric ANOVA analysis when studying the adipocyte viability (Fig. 3).

In order to confirm whether the observed decreased viability was related to adipocyte apoptosis, further experiments were carried out to determine whether the combina-

tion of antiretroviral drugs and TNF-α induced apoptosis in 3T3-L1 adipocytes. The terminal deoxynucleotidyl TUNEL assay was used to detect DNA strand cleavage, a generally accepted marker for apoptosis [18]. The TUNEL assay is used frequently as a means to detect apoptosis in a variety of cells and has been reported to detect this cellular process in the 3T3-L1 cell line [19]. Adipocytes at day 9 of the differentiation protocol were treated with vehicle or antiretroviral drugs in the presence, or absence, of TNF-α 10 ng mL^{-1}. Three days after the onset of treatment, cells were assayed for TUNEL reactivity by flow cytometry. Positive staining for TUNEL was detected in less than 10% of adipocytes exposed to the vehicle alone (Fig. 4). The same approach, applied to study the adipocyte viability, was also used to assess the TUNEL reactivity and obtained similar results ($P < 0.0001$ for both the drug treatment and the TNF-α factors) (Fig. 4). In the latter two analyses, the interaction between drug treatment and TNF-α was never significant ($P = 0.3673$ for viability and $P = 0.1081$ for TUNEL experiments). These analyses indicate that TNF-α is clearly associated to the adipocyte viability and TUNEL reactivity, independently of the drug treatment exposition.

Discussion

The clinical part of this study had two main findings; firstly, subcutaneous adipocyte apoptosis is significantly increased
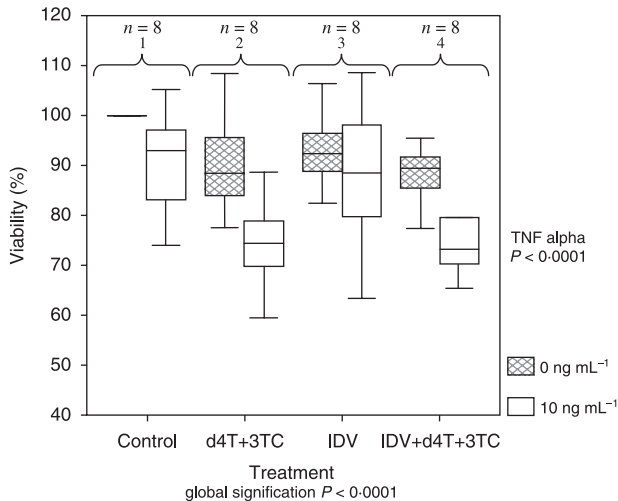
Figure 3 Effects of antiretroviral drugs and TNF-α on viability of adipocytes. Cell viability was assessed by staining with MTT and expressed relative to cells incubated in medium containing antiretroviral drugs or their combination at an individual concentration of 20 μM for 72 h, without (▨) or with (☐) TNF-α (10 ng mL^{-1}). Significant Bonferroni-adjusted post-hoc comparisons: Control (1) vs. d4T+3TC (2), $P = 0.0001$; Control (1) vs. IDV (3), $P = 0.0241$; Control (1) vs. IDV+d4T+3TC (4), $P = 0.0001$; and d4T+3TC (2) vs. IDV (3), $P = 0.0316$. TNF-α = tumour necrosis factor alpha, IDV = indinavir, d4T = stavudine, 3TC = lamivudine.
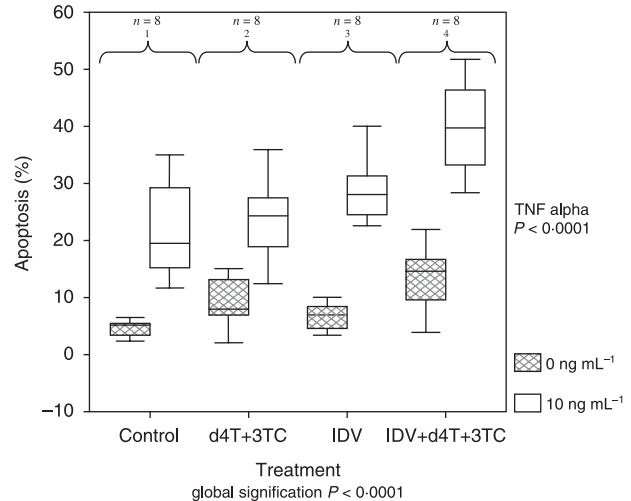
Figure 4 Effects of antiretroviral drugs and TNF-α on adipocyte apoptosis. Apoptosis was detected by TUNEL staining and flow cytometry analysis. Adipocytes were cultured in DMEM medium containing antiretroviral drugs or their combination at an individual concentration of 20 μM for 72 h, without (▨) or with (☐) TNF-α (10 ng mL^{-1}). Significant Bonferroni-adjusted post-hoc comparisons: Control (1) vs. IDV (3), $P = 0.0307$; Control (1) vs. IDV+d4T+3TC (4), $P = 0.0001$; and d4T+3TC (2) vs. IDV+d4T+3TC (4), $P = 0.0001$. TNF-α, tumour necrosis factor alpha, IDV, indinavir, d4T, stavudine, 3TC, lamivudine.

in HIV-1-infected patients with FRS, when compared with those with no FRS and the uninfected controls; and secondly, a significant correlation between serum levels of soluble TNF-α receptors and the degree of adipocyte apoptosis was observed. On the other hand, the study has been unable to establish any correlation between adipocyte apoptosis and serum levels of leptin or interleukin-6. Therefore, these findings show that a positive correlation exists between the degree of TNF-α system activation and the severity of subcutaneous adipocyte apoptosis, which in turn is the hallmark of the lipoatrophy observed in the clinical setting [1,2]. Although these associations do not prove causality, the study observed that the addition of TNF-α to an adipocyte culture embedded with antiretrovirals significantly decreased adipocyte viability and increased adipocyte apoptosis. The findings in 3T3-L1 adipocytes show that although the combination of antiviral drugs can exert a significant pro-apoptotic effect, the presence of TNF-α causes an increase in adipocyte apoptosis independently of the presence of antiretrovirals.

However, these results should be considered in the light of their inherent limitations. Firstly, the cross-sectional study design complicates drawing causal inferences from among the associations described. A single assessment of some plasma measures may be susceptible to substantial short-term variation, which could lead to attenuation of the relationship between the levels of sTNFRs and the degree of adipocyte apoptosis. To overcome such a limitation, cytokine levels were always measured when patients were

free of concomitant infections or inflammatory conditions. This research has focused on the role of TNF-α, but other cytokines may also have played a role in either favouring, or counteracting, the effects of TNF-α on the adipocyte [20]. Although the experimental part of this research strongly argues in favour of the role of TNF-α, the absence of an *in vitro* similar cellular environment to that found *in vivo* is a factor that may make it difficult to extrapolate these data to the events that occur *in vivo*.

Available data from *in vitro* and *ex vivo* studies suggest that IL-6 production by adipocytes exposed to antiretrovirals is increased [7,23,25], although to a variable extent, ranging from significant increases [23] to non-significant ones [24–26]. Regarding plasma IL-6 levels, the different reports that have assessed it have offered inconsistent data. Two studies reported that FRS+ had significantly increased plasma IL-6 levels [25,27], but at least four studies have found that plasma IL-6 levels were similar in FRS+ and FRS– patients [24,26,28,29]. Hence, these data are concordant with that reported in the literature by several authors, taking also into account that the greater BMI of our controls could have accounted for the difference the study found. However, it appears that there is an increased production of IL-6 in adipose tissue in the setting of a lipodystrophy, but this local excess of production is not always accompanied by a systemic increase of IL-6. Therefore, it can be suggested that the excess IL-6 production in adipose tissue acts as autocrine/paracrine rather than in an endocrine manner.

The TNF-α homeostasis is profoundly altered by HIV-1 infection itself and it has been shown that HIV-1-infected patients have elevated pretreatment levels of both TNF-α and its receptors and that these levels dramatically decrease when HAART is started [30]. However, concentrations of the components of TNF-α system are not normalized when HAART is effective [25,30]. There are at least two mechanisms that may contribute to an increased production of TNF-α in the setting of HIV-1-infected patients successfully treated with HAART: an excessive production of TNF-α in subcutaneous adipose tissue and the progressive accumulation in the blood of TNF-α T cell producers. In cultures of adipocytes obtained from patients with FRS, an increased secretion of TNF-α has been found [25]. Indeed, this production of TNF-α appears to be induced by protease inhibitors [21]. Bastard *et al.* [31] found that TNF-α mRNA was up-regulated in subcutaneous adipocytes from patients with FRS. Moreover, it has been recently found that TNF-α and IL-6 expression in subcutaneous adipose tissue correlated positively with the level of adipocyte apoptosis [22]. When histopathologic studies of adipose tissue from lipoatrophic areas of HIV-infected patients have been performed, lipogranulomas are a constant finding which suggests an excess local TNF-α production [1,32,33]. In a recent study, the influence of HAART on TNF-α was investigated [6], where the natural progression of HIV-1 infection, susceptibility to apoptosis of TNF-α producers, progressively increased and this was correlated with its decreased representation in the patients' blood [34]. Under HAART, apoptosis in TNF-α T-cell producers was highly suppressed, leading to its progressive accumulation in the blood [34]. These findings, of significantly higher increases in CD8 T cells in FRS+ patients, point in the same direction. Similarly, other authors [35,24,25] have shown that soluble TNF-α receptors #1 and #2 were significantly elevated, in amounts comparable to those of our patients, in HIV-1-infected patients with FRS relative to patients without FRS. In addition, circulating levels of TNF-α correlated negatively with adiponectin levels [24].

All these observations suggest that HAART creates a pro-inflammatory environment that might contribute to the development of FRS. This may be favoured in certain genetically predisposed patients because the association of a polymorphism, in position 238 G/A in the TNF-α promoter region gene, has been reported in HIV-infected patients with FRS [36,37]. This polymorphism may have functional implications as it may cause, in some patients, a polarization to TNF-α production.

TNF-α has numerous and important roles in adipose tissue metabolism [20,38]. It directly induces insulin resistance in adipocytes *in vitro* via a number of mechanisms: it inhibits insulin receptor tyrosine kinase activity by production of a serine phosphorylated (inhibitory) insulin receptor substrate-1 [39] and it down–regulates mRNA for the insulin-sensitive glucose transporter GLUT4 [40]. Additionally, TNF-α has a dose-dependent lipolytic effect on adipocytes [40], in part by down-regulating the production and activity of the enzyme lipoprotein lipase [41]. Finally, *in vitro* studies have demonstrated that TNF-α impairs human pre-adipocyte differentiation, promotes de-differentiation of mature adipocytes [42] and induces apoptosis of adipocytes and preadipocytes [5]. The suppressive effects of TNF-α on adipogenesis are exacerbated by the concomitant presence of HIV-1 protease inhibitors [43]. The potential net effect of all these influences is to reduce adipose mass by mediating a decrease in both adipocyte volume (insulin resistance, lipolytic effects) and adipocyte number (antidifferentiation, de-differentiation effects, apoptosis). As adipose tissue reflects the number and average volume of adipose cells, TNF-α may be a major contributor to adipose tissue loss.

In summary, the clinical and experimental findings suggest that an excessive amount of TNF-α may play an important role in adipocyte loss owing to apoptosis that is in turn a relevant event in the pathogenesis of FRS associated with HIV-1 infection and HAART.

Acknowledgements

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ESTUDIO 2

ORIGINAL ARTICLE AND META-ANALYSIS

No relationship between TNF-α genetic variants and combination antiretroviral therapy-related lipodystrophy syndrome in HIV type 1-infected patients: A case-control study and a meta-analysis.

Sergi Veloso, Montserrat Olona, Joaquim Peraire, Consuelo Viladés, Pedro Pardo, Pere Domingo, Victor Asensi, Montserrat Broch, Carmen Aguilar, Miguel López Dupla, Gerard Aragonés, Graciano García Pardo, Joan Josep Sirvent, Joan Vendrell, Cristóbal Richart and Francesc Vidal

No Relationship Between *TNF-α* Genetic Variants and Combination Antiretroviral Therapy-Related Lipodystrophy Syndrome in HIV Type 1-Infected Patients: A Case-Control Study and a Meta-Analysis

Sergi Veloso,[1,2,3] Montserrat Olona,[1,2,3] Joaquim Peraire,[1,2,3] Consuelo Viladés,[1,2,3] Pedro Pardo,[2,3,4]
Pere Domingo,[5,6] Victor Asensi,[7,8] Montserrat Broch,[2,3,9] Carmen Aguilar,[2,3,9] Miguel López-Dupla,[1,2,3]
Gerard Aragonés,[2,4] Graciano Garcia-Pardo,[1,2,3] Joan-Josep Sirvent,[1,2,3] Joan Vendrell,[1,2,3,10]
Cristóbal Richart,[1,2,3,9] and Francesc Vidal,[1,2,3] for the HIV Lipodystrophy Study Group

Abstract

Tumor necrosis factor alpha (TNF-α) is thought to be involved in the pathogenic and metabolic events associated with HIV-1 infection. We assessed whether carriage of the *TNF-α* gene promoter single nucleotide polymorphism (SNP) is associated with lipodystrophy and metabolic derangements in HIV-1-infected patients treated with cART. We also assessed variations in TNF-α receptor plasma levels. The study group comprised 286 HIV-1-infected patients (133 with and 153 without lipodystrophy) and 203 uninfected controls (UC). *TNF-α −238G > A,* *−308G > A,* and *−863 C > A* SNP were assessed using PCR-RFLPs on white cell DNA. Plasma sTNF-α R1 and R2 levels were measured by ELISA. Student's *t* test, the χ^2 test, Pearson correlations, and the logistic regression test were performed for statistical analysis. The *TNF-α −308G > A* SNP was significantly associated with lipodystrophy in the univariate analysis ($p = 0.04$). This association, however, was no longer significant in the multivariate analysis. A meta-analysis of the published literature and our own data, which included 284 patients with lipodystrophy and 338 without lipodystrophy, showed that there was no relationship between the *TNF-α −238G > A* and *−308G > A* SNP and lipodystrophy ($p > 0.05$ for all comparisons). HIV-1-infected patients had greater sTNF-α R2 plasma levels than UC ($p = 0.001$) whereas sTNF-α R1 and R2 levels were not significantly different in both the HIV-1-infected cohorts, lipodystrophy vs. nonlipodystrophy ($p = $ NS). In our cohort of white Spaniards the *TNF-α −238G > A,* *−308G > A,* and *−863C > A* SNP were not associated with lipodystrophy in HIV-1-infected patients treated with cART. This finding was replicated in a meta-analysis of the published data, which showed no associations between the *TNF-α −238G > A* and *−308G > A* SNP and lipodystrophy. In HIV-1-infected patients under cART there is a systemic overproduction of sTNF-α R2, which is unrelated to the presence of lipodystrophy.

Introduction

TUMOR NECROSIS FACTOR (TNF)-α is a pleiotropic cytokine that acts as an immune and inflammatory mediator. It is synthesized mainly by macrophages and T cells and most of its actions take place by binding to two cell receptors: TNF-α R1 and R2.[1] In adipose tissue, TNF-α promotes insulin resistance, inhibits adipogenesis, causes lipolysis as well as apoptosis of preadipocytes and mature adipocytes, and stimulates the conversion of preadipocytes into

[1]Hospital Universitari de Tarragona Joan XXIII, Tarragona, Spain.
[2]IISPV, Tarragona, Spain.
[3]Universitat Rovira i Virgili, Tarragona, Spain.
[4]Hospital Universitari de Sant Joan, Reus, Spain.
[5]Hospital de la Santa Creu i Sant Pau, Barcelona, Spain.
[6]Universitat Autònoma de Barcelona, Barcelona, Spain.
[7]Hospital Universitario Central de Asturias, Oviedo, Spain.
[8]Universidad de Oviedo, Oviedo, Spain.
[9]CIBER Fisiopatologia de la Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III, Madrid, Spain.
[10]CIBER Diabetes y Enfermedades Metabólicas Asociadas (CB07/08/0012), Instituto de Salud Carlos III, Madrid, Spain.

macrophages, thus perpetuating inflammation.[2–4] Because most of these features are found in the combination anti-retroviral (cART) -related lipodystrophy syndrome, various studies have assessed and confirmed the involvement of TNF-α in this process.[5–12]

As with many other proinflammatory cytokines, TNF-α production is at least partially genetically determined.[13,14] Several functional single nucleotide polymorphisms (SNP) have been identified within the *TNF-α* gene cluster.[13–17] The most widely studied are a $G > A$ transition at position -238, a $G > A$ transition at position -308, and a $C > A$ transition at position -863.[18–20] Several cohort studies have assessed the influence of *TNF-α* SNP on the vulnerability to lipodystrophy and its associated metabolic derangements in HIV-1-infected patients treated with cART.[21–24] Although some studies have shown a relationship between the carriage of the rare *TNF-α* $-238A$ allele with both the risk[21] and rapidity[22] of developing lipodystrophy, other investigations have failed to do so.[23,24]

Given these inconsistencies, we undertook the present study in a large cohort of white Spanish HIV-1-infected patients treated with cART with and without lipodystrophy and in an uninfected control (UC) group. Our objectives were to assess whether the *TNF-α* SNP are associated with the risk of developing lipodystrophy and its associated metabolic derangements, and to evaluate changes in plasma TNF-α receptor levels. We also performed a meta-analysis of our own data and data from previous case-control studies in which *TNF-α* polymorphisms were analyzed in patients with lipodystrophy.[21–24]

Materials and Methods

Design, setting, and participants

This was a multicenter cross-sectional association study. We evaluated 489 persons: 286 adult HIV-1-infected patients and 203 UCs. Patients were recruited from a prospectively collected cohort of 1700 HIV-1-infected individuals who were receiving cART, defined as the combination of two nucleoside reverse transcriptase inhibitors (NRTIs) plus either protease inhibitors (PIs) or nonnucleoside reverse transcriptase inhibitors (NNRTIs). The patients were followed up at the HIV outpatient clinic of the three participating hospitals. Inclusion criteria were older than 18 years, the presence of HIV-1 infection, stable cART regimen for at least 1 year, and the presence or absence of lipodystrophy according to a predefined criteria (see below). We recruited all those patients who fulfilled the criteria of lipodystrophy and who agreed to participate in the study ($n = 133$) as well as a randomly selected group of patients without lipodystrophy ($n = 153$) whose age, gender, and time of exposure to cART were comparable to that of patients with lipodystrophy.[25,26] The control group was made up of uninfected healthy persons comparable to the patients in age and gender. Individuals with HIV-1-associated cachexia, active opportunistic infections, current inflammatory diseases, and those taking drugs with known metabolic effects such as steroids and hormones were excluded from the study, as were individuals with plasma C reactive protein > 1 mg/dl. All subjects were white Spaniards. Immigrants and their descendants (including those from other European countries) were excluded. All participants gave informed consent. The project was approved by the local ethical committees.

Assessment of lipodystrophy

All HIV-1-infected patients had a complete physical examination during which the type (lipoatrophy or mixed) and degree (slight, moderate, or severe) of lipodystrophy were assessed. Patients with pure lipohypertrophy ($n = 4$) were excluded. Waist and hip diameter, height, weight, and body mass index (BMI) were measured. The presence of lipodystrophy was defined by changes in the body fat composition that were important enough to be recognized by both the patient and the attending physician. Lipoatrophy was defined by the presence of one or more of the following criteria: loss of fat from the face, arms, and legs, prominent veins in the arms and legs, and a thin bottom. Lipohypertrophy was defined by the presence of one or more of the following criteria: an increase in the abdominal perimeter, breast, and/or neck fat deposition. We determined mixed lipodystrophy when at least one characteristic of lipoatrophy and one of lipohypertrophy were concomitantly present in one patient. Lipodystrophy was categorized according to the scale proposed by Carr *et al.*[27] as nonexistent (0), slight (1), moderate (2), or severe (3). Doubtful cases were excluded. This categorization was evaluated in the face, arms, legs, buttock, abdomen, neck, and breasts. The sum of the values for each body zone indicated the degree of lipodystrophy: none (0), slight (1–6), moderate (7–12), and severe (13–18).[25–28] In this study only moderate and severe cases were included in order to avoid superposition between groups. To objectively assess the distribution of subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), a single-slice computed tomography (CT) scan at the level of L4 was made of in all the participants in the study. The surface of adipose tissue was measured in cm^2.

Blood, DNA, and plasma samples

After 12 h fasting, blood samples with ethylenediaminetetraacetic acid (EDTA) were obtained from an antecubital vein. Five milliliters of whole blood was used to determine CD4$^+$ T cell count, and 500 μl was used for DNA isolation by a MagNa Pure LC Instrument (Roche Diagnostics, Basel, Switzerland). Plasma and serum were obtained by centrifugation at $3500 \times g$ for 15 min at 4°C and were stored at -80°C until use.

Laboratory methods

Blood chemistry. Serum glucose was measured using the glucose oxidase method with a Hitachi autoanalyzer. Lipid profile (serum total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides) was measured using the usual enzymatic methods. Hyperglycemia was defined as a blood glucose level of ≥6.1 mmol/liter, hypertriglyceridemia was defined as a triglyceride level of ≥2.2 mmol/liter, hypercholesterolemia was defined as a total cholesterol level of ≥5.2 mmol/liter, a low HDL-cholesterol was defined as a level of ≤0.9 mmol/liter, and a high LDL-cholesterol was defined as a level of ≥3.4 mmol/liter.[29] Hyperinsulinemia was considered when insulin levels were ≥18 μU/ml.[30] Insulin resistance was calculated using to the homeostasis model assessment for insulin resistance (HOMA-r) method from fasting glucose and insulin concentrations, using the following formula: insulin (μIU/ml) × glucose (mmol/liter)/22.5.

HIV-1 infection. This was diagnosed using an enzyme immunoanalysis and confirmed by a Western blot test.

HCV infection. This was assessed by detecting anti-HCV antibodies in serum, through indirect qualitative immunoassay (sandwich washed twice) (Advia Centaur, Bayer Health Care, Tarrytown, NY).

Plasma HIV-1 viral load. This was determined by the HIV Cobas Ampliprep CAP-CTMHIV-1 using the COBAS AMPLICOR system (Roche Diagnostics, Basel, Switzerland). The cutoff for undetectable viral load was 200 copies/μl.

Assessment of blood CD4^{+} T cell lymphocytes. Samples were analyzed in a flow cytometer FAC Scan (Becton Dickinson Immunocytometry Systems, San José, CA). The data acquired were analyzed using the Multiset program.

TNF-α $-238G > A$, $-308G > A$, and $-863C > A$ genotypes. These were assessed by restriction fragment-length polymorphism as previously described in detail by our group.[18,19]

Plasma sTNF-α R1 and R2 levels. These were used to assess systemic TNF-α production and were determined by solid-phase enzyme-amplified sensitivity immunoassays on a microtiter plate (MEDGENIX sTNF-R1 and sTNF-R2 EASIA; BioSource Europe S.A., Fleunes, Belgium), as we previously described.[19]

Statistical analysis

Continuous variables were expressed as mean $\pm$ SD. Prior to statistical analyses, normality distribution and homogeneity of the variances were tested. Student's t test was used to compare continuous variables between two groups. Qualitative variables, including genotype and allele frequencies were analyzed by the χ^2 test. The Hardy–Weinberg equilibrium was assessed by the χ^2 goodness-of-fit test. The relationship between plasma TNF-α levels and other continuous variables was assessed by Pearson correlation analyses. The association between potential factors and the presence of lipodystrophy and metabolic alterations was examined by forward stepwise logistic regression analysis and the model was adequately fitted by the Hosmer and Lemeshow's goodness of fit test. The variables included in the multivariate analysis were those that had presented a p-value of less than 0.2 in the univariate analysis and those that were biologically plausible. All analyses were performed using the SPSS/PC + statistical package (V. 11.01 for Windows; Chicago, IL). A p-value of <0.05 was considered significant.

Meta-analysis

All studies published before 2009 were identified through a PubMed database search, using the following keywords: tumor necrosis factor alpha, polymorphism, HIV, lipodystrophy. A subsequent manual search was performed using the references from the selected articles. Case-control studies that assessed the distribution of the TNF-α genetic variants in HIV-1-infected patients with and without lipodystrophy were eligible for inclusion in the meta-analysis,[21–24] which also included our own data. Cases were defined as HIV-1-infected patients treated with cART who had lipodystrophy. Controls were defined as HIV-1-infected patients under cART without lipodystrophy. The criteria and categorization for the presence or absence of lipodystrophy were those used by the authors.[21–24] Genotype and allele frequencies were extracted or calculated from the published data. In some cases, authors were contacted and asked to provide additional data.[24] The bibliography was searched and the data extracted independently by two authors (M.O. and F.V.) and consensus was reached for all data. An exploratory meta-analysis was carried out to assess the association between carriage of the TNF-α $-238A$ and $-308A$ variant alleles and the risk of lipodystrophy. The OR and its 95% CI were estimated for each gene. Cochran's Q-statistic was used to assess heterogeneity across studies: a significant Q-statistic ($p < 0.1$) indicated heterogeneity, and therefore the DerSimonian and Laird random model was used for meta-analysis; otherwise the Mantel–Haenszel fixed effect model was used. A sensitivity analysis was performed to assess the effect of excluding specific studies.[31,32] The meta-analysis was performed using the computer software package RevMan 4.2.8.[33]

Results

Characteristics of the participants

HIV-1-infected patients and UC were comparable in age (42 ± 11.9 vs. 42 ± 8.9; $p = 0.7$) and gender (66.1% vs. 64.4% male; $p = 0.7$). Table 1 shows the main characteristics of the HIV-1-infected patients studied, categorized according to the presence or absence of lipodystrophy. Of the 133 HIV-1-infected subjects with lipodystrophy, 31 (23%) had pure lipoatrophy and 102 (77%) had a mixed form. Compared to those infected with HIV-1 but without lipodystrophy, the group with lipodystrophy had a significantly greater BMI, waist/hip circumference ratio, a significantly lower SAT, and a greater VAT. Patients with lipodystrophy had a more advanced disease defined by the CDC classification and a greater CD4^{+} T cell gain due to cART, compared with those without lipodystrophy. Also, patients with lipodystrophy had consumed a greater number of PIs and NRTIs and had, on the whole, been exposed longer to NRTIs and, particularly, to stavudine (d4T) (Table 1).

Biochemical data

Table 2 shows the main biochemical data of the HIV-1-infected patients categorized according to the presence or absence of lipodystrophy. No biochemical data were available from the UC cohort. Glucose, insulin, triglyceride, total and LDL-cholesterol plasma levels, and HOMA-IR were significantly increased in patients with lipodystrophy in comparison to those without.

Plasma sTNF-α R1 and R2 levels in HIV-1-infected patients with lipodystrophy were slightly but nonsignificantly greater than in patients without lipodystrophy ($p = $ NS). Values were lowest in the uninfected population (Table 2). The possible relationship between the plasma TNF-α R1 and R2 levels and metabolic outcomes was evaluated. Variables included in the multivariate analysis were age, AIDS stage, duration of HIV-1 infection, consumption and cumulative time of NRTIs, NNRTIs, and PIs, CD4^{+} T cell gain, BMI > 25, and plasma sTNF-α R1 and R2 levels. On the whole infected cohort, the only independent significant associations were between plasma sTNF-α R1 and R2 levels and plasma total cholesterol/hypercholesterolemia (OR: 1.82, 95% CI: 1.01–3.3, $p = 0.04$ and

Table 1. Characteristics of the HIV-1-Infected Subjects Studied Categorized According to the Presence or Absence of Lipodystrophy[a,b]

Variable	HIV-1 infected (n = 286)		p Value
	Without lipodystrophy (n = 153)	With lipodystrophy (n = 133)	
Age (years)	41 ± 7.6	43.2 ± 10.1	0.039
Male (%)	67.9	60.2	0.17
Body mass index (kg/m^2)	22.8 ± 3.1	23.6 ± 2.8	0.042
Body mass index > 25 (%)	20.3	30.3	0.072
Waist/hip circumference ratio	0.89 ± 0.1	0.91 ± 0.1	0.05
SAT (cm^2)[c]	127.4 ± 60.9	49.9 ± 30.7	0.012
VAT (cm^2)[c]	37.2 ± 26.9	109.5 ± 66	0.004
AIDS (A3, B3, C category) (%)	37.4	52.7	0.025
HIV-1 risk factor, (%)			
Injection drug user	44.4	32.6	0.049
Homosexual	19.9	18.6	0.87
Heterosexual	35.7	43.4	0.22
Other/unknown	0	5.4	0.004
HCV infection (%)	55.3	44.4	0.114
Duration of HIV infection (years)	8.7 ± 4.2	9.7 ± 4.6	0.069
CD4$^+$ T cell count (cells/ml)			
Nadir	319.5 ± 267.7	254.6 ± 206.4	0.028
Current	523.6 ± 309.1	579.7 ± 328.1	0.139
CD4 recovery (current-nadir CD4)	196 ± 307.1	325.2 ± 319.1	0.001
Plasma HIV-1 RNA			
Pre-cART (copies/ml)	275902.5 ± 634189.7	216959.7 ± 394712.9	0.385
Current plasma viral load < 200 (%)	76.3	81.2	0.31
Antiretroviral therapy (months)[d]			
Duration of cART	60.8 ± 29.4	53.6 ± 22.9	0.082
Exposure to NRTIs before cART, yes (%)	34.1	54	0.050
Cummulative time on NRTIs	99.8 ± 60.3	121.7 ± 49.9	0.001
NRTI consumption, yes (%)	94.1	100	0.004
Cummulative time on d4T	14.7 ± 20.6	34.7 ± 21.9	<0.001
Cummulative time on AZT	33.1 ± 30	26.2 ± 25.9	0.04
Cummulative time on ddC	3 ± 9.6	7.7 ± 13.6	0.001
Cummulative time of PI	30.3 ± 29.4	33.3 ± 23.8	0.485
PI consumption, yes (%)	73.9	84.1	0.043
Cummulative time on NNRTIs	14.8 ± 17.5	17.2 ± 16.7	0.233
NNRTI consumption, yes (%)	68.4	72.2	0.518

[a]Quantitative variables are expressed as mean ± standard deviation. Qualitative variables are expressed as percentage.

[b]SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; cART, combination antiretroviral therapy; NRTIs, nucleoside reverse transcriptase inhibitors; d4T, stavudine; AZT, zidovudine; ddC, zalcitabine; PIs, protease inhibitors; NNRTIs, nonnucleoside reverse transcriptase inhibitors; ddI, didanosine. 3TC, lamivudine; ABC, abacavir; TFV, tenofovir; EFV, efavirenz; NVP, nevirapine; SQV, saquinavir; IDV, indinavir; RTV, ritonavir; NFV, nelfinavir; APV, amprenavir; LPV, lopinavir; ATV, atazanavir.

[c]Measured by CT scan performed al the level of L4.

[d]Use of some other antiretroviral drugs (ddI, 3TC, ABC, TFV, EFV, NVP, SQV, IDV, RTV, NFV, APV, LPV, ATV) was nonsignificantly different between groups.

OR: 1.2, 95% CI: 1.01–1.04, $p = 0.04$, respectively), plasma LDL-cholesterol/hyper-LDL-cholesterolemia (OR: 2.2, 95% CI: 1.00–4.95, $p = 0.05$ and OR: 1.4, 95% CI: 1.02–1.9, $p = 0.04$, respectively), and plasma HDL-cholesterol/hypo-HDL-cholesterolemia (OR: 0.42, 95% CI: 0.22–0.82, $p = 0.01$ and OR: 0.79, 95% CI: 0.64–0.94, $p = 0.007$, respectively). These results did not change when *TNF-α* genotypes were included in the analysis. Also, these statistically significant associations were maintained when we analyzed the subsets with and without lipodystrophy separately.

TNF-α −238G > A, −308G > A, and −863C > A SNP

The distribution of the three *TNF-α* SNP in the different groups assessed is shown in Table 3. The genotype distribu-

tion in uninfected subjects, HIV-1-infected with lipodystrophy, and HIV-1-infected without lipodystrophy fits the expected Hardy–Weinberg equilibrium for each *TNF-α* SNP. The effect of *TNF-α* genotypes on the development of lipodystrophy and on the metabolic outcomes studied was evaluated. Associations with lipodystrophy indicated that the *TNF-α −308A* genetic variant was significantly overrepresented in patients without lipodystrophy, but in allele-wise comparisons only ($p = 0.04$) (Table 3). This association, however, was no longer observed when a multivariate analysis was performed using the following variables: age, BMI more than 25, waist-to-hip circumference ratio, AIDS stage, risk factor for HIV infection, duration of HIV-1 infection, CD4$^+$ T cell gain, duration of cART, consumption of NRTIs previous to cART, consumption and cumulative time of PIs, NRTIs,

TABLE 2. BLOOD CHEMISTRY AND PLASMA sTNF-R1 AND sTNF-R2 LEVELS IN THE COHORT STUDIED[a]

Variable	UC (n = 203)	HIV-1-infected Without lipodystrophy (n = 153)	With lipodystrophy (n = 133)	p-value
Glucose/insulin				
Glucose (mmol/liter)		5.2 ± 0.7	5.5 ± 1.4	0.02
Hyperglicaemia ≥6.1 (%)		11.2	19.5	0.06
Insulin (μU/ml)	—	8.9 ± 9.9	15.9 ± 18.2	<0.001
Insulin ≥18, (%)		10.5	29.7	<0.001
HOMA-IR		3.1 ± 3.4	4.8 ± 5.4	0.032
Lipids (mmol/liter)				
Triglyceride		1.8 ± 1.4	2.9 ± 2.2	<0.001
Triglyceride ≥ 2.2 (%)		23	50.8	<0.001
Total cholesterol		4.9 ± 1.2	5.5 ± 1.4	0.001
Total cholesterol ≥5.2 (%)	—	40.1	55.6	0.012
LDL-cholesterol		2.8 ± 1	3.3 ± 1.1	0.001
LDL-cholesterol ≥ 3.4 (%)		25.7	43.8	0.003
HDL-cholesterol		1.3 ± 0.6	1.1 ± 0.4	<0.001
HDL-cholesterol ≤0.9 (%)		19.2	36.4	0.001
sTNF-R1 (ng/ml)	2.02 ± 0.76	2.11 ± 0.98	2.52 ± 3.36	0.55#
sTNF-R2 (ng/ml)	3.76 ± 2.13	6.73 ± 4.06	6.98 ± 5.17	<0.0001[b]

[a]Quantitative variables are expressed as mean ± standard deviation. Qualitative variables are expressed as percentage. UC, uninfected controls.
[b]p-values between UC and HIV-1-infected patients (p = NS between HIV patients with and without lipodystrophy).

NNRTIs, d4T, and AZT, HCV infection, sTNF-α R1 and R2 plasma levels, and *TNF-α* genotype. No association was found between *TNF-α* genotypes and the severity of lipodystrophy (moderate or severe): *TNF-α* −238, $p = 0.2$; −308, $p = 0.1$; −863, $p = 0.22$. As far as metabolic outcomes were concerned, there was a significant association between carriage of the *TNF-α* −863AA homozygous variant genotype and HOMA-r in patients with lipodystrophy ($p = 0.02$), which was inde-

pendent of the type of antiretroviral drugs that the patients received (NRTIs, NNRTIs, or PIs).

We also evaluated the effect of the *TNF-α* genotypes on the vulnerability to HIV-1 infection. The distribution of the *TNF-α* −308G > A and −863C > A SNP was significantly different in uninfected and infected subjects. Carriers of the −308A and −863A variant alleles were significantly overrepresented in uninfected subjects, and this was observed in both the

TABLE 3. GENOTYPE DISTRIBUTION AND ALLELE FREQUENCIES OF *TNF-α* −238G > A, −308G > A, AND −863C > A SNP IN UNINFECTED CONTROLS AND IN HIV-1-INFECTED SUBJECTS CATEGORIZED ACCORDING TO THE PRESENCE OR ABSENCE OF LIPODYSTROPHY[a]

Genotype and allele frequencies	ALL UC (n = 203)	HIV infected (n = 286)	p Value	HIV-1 infected Without lipodystrophy (n = 153)	With lipodystrophy (n = 133)	p Value
TNF −238 G > A						
GG	143 (87.2%)	228 (87.7%)		121 (87.7%)	107 (87.7%)	
GA	20 (12.2%)	30 (11.5%)	0.96	16 (11.6%)	14 (11.5%)	0.99
AA	1 (0.6%)	2 (0.8%)		1 (0.7%)	1 (0.6%)	
GA + AA	21 (12.8%)	32 (12.3%)	0.96	17 (12.3%)	15 (11.3%)	0.9
Rare allele A	22 (6.7%)	34 (6.5%)	0.86	18 (6.5%)	16 (6%)	0.86
TNF −308 G > A						
GG	122 (70.9%)	209 (82.6%)		103 (79.2%)	106 (86.2%)	
GA	42 (24.4%)	37 (14.6%)	0.017	21 (16.2%)	16 (13%)	0.13
AA	8 (4.7%)	7 (2.8%)		6 (4.6%)	1 (0.8%)	
GA + AA	50 (29.1%)	44 (17.4%)	0.007	27 (20.8%)	17 (13.8%)	0.14
Rare allele A	58 (16.9%)	51 (11.2%)	0.006	33 (12.7%)	18 (5.5%)	0.04
TNF −863 C > A						
CC	116 (69.9%)	192 (83.1%)		93 (79.5%)	99 (86.8%)	
CA	44 (26.5%)	31 (13.4%)	0.004	19 (16.2%)	12 (10.5%)	0.328
AA	6 (3.6%)	8 (3.5%)		5 (4.3%)	3 (2.6%)	
CA + AA	50 (30.1%)	39 (20.5%)	0.0003	24 (20.5%)	15 (13.1%)	0.14
Rare allele A	56 (16.8%)	47 (11.3%)	0.001	29 (12.4%)	18 (7.9%)	0.11

[a]Results are expressed as n (%). UC, uninfected controls. Genotype distribution and allele frequencies in the UC and HIV-1-infected subjects were in accordance with the expected Hardy–Weinberg equilibrium. Genotype and allele numbers do not match with participants because DNA for *TNF-α* genotyping was not available or could not be amplified in some individuals.

Table 4. Characteristics of the Studies Considered in the Meta-analysis of the $TNF\text{-}\alpha\ -238G > A$ and $-308G > A$ in cART-Related Lipodystrophy in HIV-1-Infected Patients[a]

Reference	Selection criteria of cases and controls and genotype distribution
Maher et al.[21]	Cases: HIV-1-infected patients treated with cART with lipodystrophy ($n = 61$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 52, GA 8, AA 1 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG 45, GA 12, AA 4 Controls: HIV-1-infected patients treated with cART without lipodistrophy ($n = 35$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 35, GA 0, AA 0 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG 21, GA 13, AA 1
Asensi et al.[24,a]	Cases: HIV-1-infected patients treated with cART with lipodystrophy ($n = 90$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 66, GA 9, AA 0 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG58, GA22, AA 1 Controls: HIV-1-infected patients treated with cART without lipodistrophy ($n = 150$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 120, GA12, AA 0 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG 108, GA 25, AA 3
Veloso et al. (present report)[a]	Cases: HIV-1-infected patients treated with cART with lipodystrophy ($n = 133$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 107, GA 14, AA 1 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG 106, GA 16, AA 1 Controls: HIV-1-infected patients treated with cART without lipodystrophy ($n = 153$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 121, GA 16, AA 1 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG 103, GA 21, AA 6
All patients included in the meta-analysis[a]	Cases: HIV-1-infected patients treated with cART with lipodystrophy ($n = 284$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 225, GA 31, AA 2 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG 209, GA 50, AA 6 Controls: HIV-1-infected patients treated with cART without lipodystrophy ($n = 338$) 　Genotype distribution $TNF\text{-}\alpha\ -238G > A$: GG 276, GA 28, AA 1 　Genotype distribution $TNF\text{-}\alpha\ -308G > A$: GG 232, GA 59, AA 10

[a]Genotype numbers do not match with participants because DNA for $TNF\text{-}\alpha$ genotyping was not available or could not be amplified in some individuals.

genotype and allele analyses (Table 3). No significant associations were observed with respect to the $TNF\text{-}\alpha\ -238G > A$ polymorphism.

Meta-analysis of the TNF-α −238G > A and −308 G > A SNP and lipodystrophy

Four articles were found to assess the relationship between $TNF\text{-}\alpha$ SNP and lipodystrophy.[21–24] One was a longitudinal study,[22] which lacked a nonlypodystrophy group and, hence, was not included. The remaining three were case-control studies, met the search criteria,[21,23,24] and were selected for the meta-analysis in conjunction with our own data. However, since Tarr et al.[23] did not provide data on lipodystrophy, we could not include their patients in the meta-analysis. Details of the studies used for the meta-analysis are shown in Table 4. Overall, the meta-analysis assessed the relationship between the $TNF\text{-}\alpha\ -238G > A$ SNP and lipodystrophy in 284 patients with lipodystrophy and 338 without lipodystrophy, and the relationship between the $TNF\text{-}\alpha\ -308G > A$ SNP and lipodystrophy in 267 patients with lipodystrophy and 301 without lipodystrophy. The effect of the $TNF\text{-}\alpha\ -863\ C > A$ SNP was not included in the meta-analysis since it had not been assessed previously. No statistical differences were detected in the genotype and allele distribution regarding the $TNF\text{-}\alpha\ -238G > A$ and $-308G > A$ SNP in patients with and without lipodystrophy (Figs. 1 and 2).

Discussion

In this study we found that the $TNF\text{-}\alpha$ genetic variants have no impact on the susceptibility of lipodystrophy in cART-treated HIV-1-infected patients. A meta-analysis of the previously published data and our own results also indicates no relationship between $TNF\text{-}\alpha\ -238G > A$ and $-308C > A$ SNP and lipodystrophy. We also show that there is a systemic overproduction of TNF-α in infected patients under cART regardless of the presence or absence of lipodystrophy. We confirm that metabolic abnormalities are highly prevalent in lipodystrophy.[30,34–36] Finally, our study agrees with previous reports that related lipodystrophy in HIV-1-infected persons under cART to a more advanced disease defined by CDC classification, to a robust $CD4^+$ T cell gain, and to the amount of cART and, particularly, of d4T exposure.[30]

Because not all HIV-1-infected patients treated with cART develop lipodystrophy and/or metabolic perturbations, host genetic vulnerability has been suggested. Among the candidate genes involved, $APOE$,[23] $APOC$,[23] IL-1β,[24] IL-6,[25] β3-adrenergic receptor,[37] and $TNF\text{-}\alpha$[21–24] variants have been sought. In the present report we have reassessed the effect of various $TNF\text{-}\alpha$ polymorphisms on lipodystrophy and its related metabolic disturbances. The rationale behind this study is the modulating effect that some $TNF\text{-}\alpha$ SNP and TNF-α itself exert on hyperglycemia, insulin resistance, and obesity in uninfected subjects[38–40]; however, data about its influence on dyslipidemia are less conclusive.[41,42]

According to our results, the $TNF\text{-}\alpha$ genetic variants are not major modulators of the risk of developing lipodystrophy in Spanish HIV-1-infected patients treated with cART, since only a marginal association between carriage of the $TNF\text{-}\alpha\ -308A$ variant allele and protection against lipodystrophy was observed, and only in the univariate analyses. This association

A. Genotype analyses
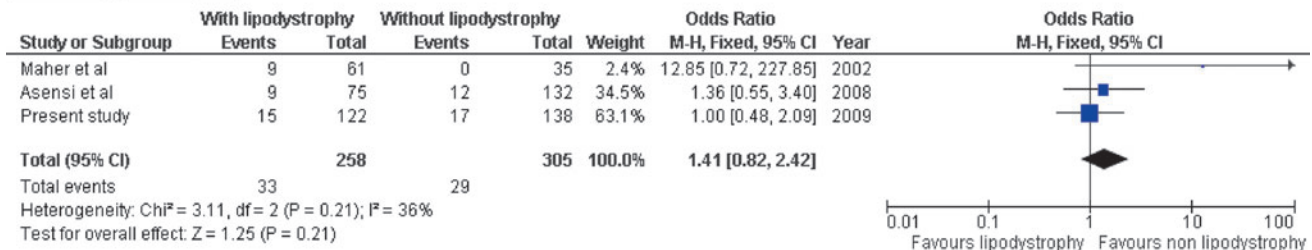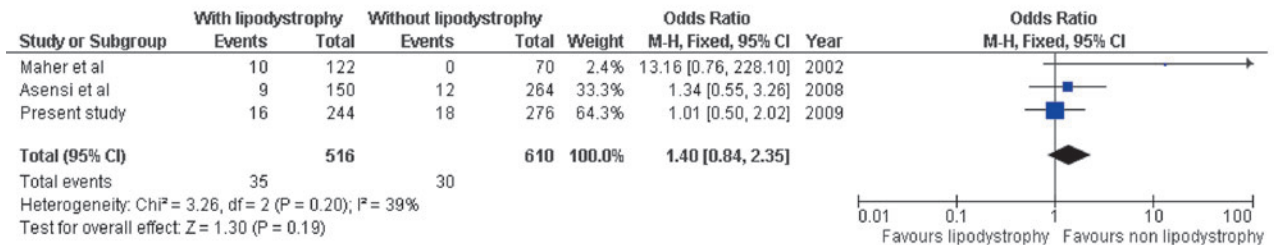
B. Allele analyses

FIG. 1. Meta-analysis to assess whether carriage of the *TNF-α −238 G > A* SNP is associated with cART-related lipodystrophy in HIV-1-infected patients. (**A**) Genotype analyses. (**B**) Allele analyses.

was no longer maintained when corrected for confounders. Few other reports have investigated the effect of TNF-α genetic variants on vulnerability to lipodystrophy[21–24] and/or to its associated metabolic disturbances[23,43] in HIV-1-infected patients treated with cART. A cross-sectional British study showed that the −238G > A SNP had a modulating effect and that the −308G > A had no role.[21] These findings were further confirmed by an Australian team in a longitudinal study.[22] However, subsequent investigations from the Swiss cohort[23] and a Spanish cohort[24] failed to detect significant associations,

A. Genotype analyses
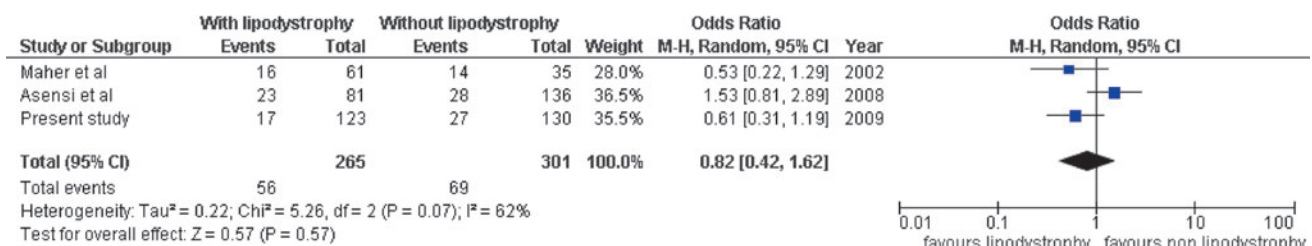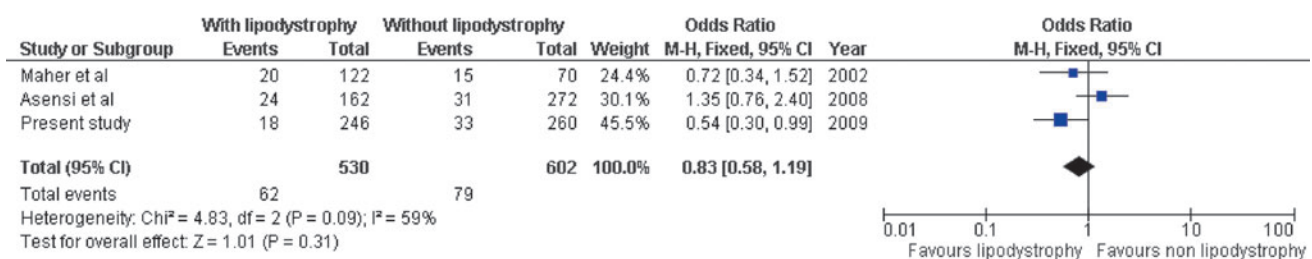
B. Allele analyses

FIG. 2. Meta-analysis to assess whether carriage of the *TNF-α −308 G > A* SNP is associated with cART-related lipodystrophy in HIV-1-infected patients. (**A**) Genotype analyses. (**B**) Allele analyses.

as was the case with our research. Because our findings are based on the assessment of a large number of persons, we believe that the lack of association with respect to the $-238G > A$ SNP is more robust than the previous report,[21] since the latter was based on the assessment of a very low number of patients with lipodystrophy and this means unstable data.[44] This does not invalidate the association between carriage of the *TNF-α −238A* variant allele and faster development of lipodystrophy reported by Nolan *et al.*[22] since their study was longitudinal and may therefore offer information different from that provided by cross-sectional investigations. The type of antiretroviral drugs that the patients received in the different cohorts may offer an additional explanation for discrepancies.[45,46] In our study we observed increased plasma levels of sTNF-α R2 in the patients, not related to lipodystrophy. Such an increase has been reported in a number of studies in patients infected with HIV.[5,11,47–49] When taken together, these data suggest that the effects of TNF-α on lipodystrophy that we and others have previously demonstrated[6,7,11] may be driven mainly by its local effects on SAT—perturbed adipocyte differentiation and increased adipocyte apoptosis—rather than by a systemic activation of TNF-α. Additional findings from our report also show that the *TNF-α* SNP assessed have no effect on hyperglycemia, or dyslipidemia in cART-treated HIV-1-infected patients with and without lipodystrophy. This finding differs from what occurs in uninfected people.[38–41]

We recognize that our study has some limitations. First, the cross-sectional nature of our design provides associations, not causality. However the present study is one of the largest ever done in the field, and this reinforces the replicability of our results. Second, our clinical definition and assessment of lipodystrophy do not allow us to discount the possibility that some nonlipodystrophy patients could have minor changes that are not clinically detectable. This raises the possibility that some patients in the nonlipodystrophy group could in fact be false negatives. We believe, however, that this is unlikely in our study because our cohort was made up of extreme lipodystrophy phenotypes and comprised only overt lipodystrophy versus clear nonlipodystrophy persons; individuals with slight/mild lipodystrophy (potential false negatives) were excluded. Also, in the subsequent clinical follow-up of our HIV-1-infected cohort, none of the patients without lipodystrophy evolved toward overt lipodystrophy.

The results of the meta-analysis indicate the neither the *TNF-α −238G > A* nor the *−308G > A* polymorphisms are associated with lipodystrophy in cART-treated HIV-1-infected patients. This tones down the weak positive association of our own study. In fact, we found positive associations regarding the *−308G > A SNP* and in the allele analysis only. Note that positive associations based on a single contrast have less chance of being replicated than associations based on more contrasts.[44] The strengths of this meta-analysis are that it provides data for 284 HIV-1-infected patients with lipodystrophy and 338 HIV-1-infected patients without lipodystrophy, hence, the data are robust and replicable. We acknowledge, however, that this meta-analysis has some weaknesses. The different definitions and categorizations of lipodystrophy in the different studies may render the lypodystrophy subset somewhat heterogeneous. We also observed in our cohort that the distribution of the *TNF-α −308G > A* and *−863C > A* SNP was significantly different

between uninfected and infected subjects. To explain the variable interindividual vulnerability to HIV-1 infection, a genetically driven host susceptibility has been suggested. Several candidate genes have been checked, one of which was *TNF-α* because its robust role in the initial response to infection influences both innate and acquired immunity.[1,50] Our data indicate that carriage of some *TNF-α* genetic variants may influence vulnerability to HIV-1 infection. These results agree with some reports that showed an association between *TNF-α* SNP and the risk of infection,[51,52] although data are inconsistent.[53–56] The discrepancy between our findings and those reported previously may be because of the low number of patients assessed in some studies, which often provides unstable data,[57] or because the different ethnicities of the people assessed in others may have led to genuine population differences.[54,55]

In summary, when taken together, the data analyzed here suggest that *TNF-α* genetic variants do not modulate the vulnerability to cART-related lipodystrophy in HIV-1-infected patients. The systemic activation of the TNF-α system reported in HIV-1-infected patients with lipodystrophy may, in fact, be related to the HIV-1 infection itself rather than to lipodystrophy. Polymorphism within the *TNF-α* gene promoter may be involved in vulnerability to HIV-1 infection.

Appendix

The members of the HIV Lipodystrophy Study Group and co-authors of this paper are Verónica Alba, Alba Aguilar, Carmen Aguilar, Teresa Auguet, Montserrat Broch, Graciano Garcia-Pardo, Frederic Gómez-Bertomeu, Miguel López-Dupla, Montserrat Olona, Joaquim Peraire, Cristóbal Richart, Amadeu Saurí, Joan-Josep Sirvent, Antoni Soriano, Montserrat Vargas, Ignacio Velasco, Sergi Veloso, Joan Vendrell, Francesc Vidal, Consuelo Viladés (Hospital Universitari Joan XXIII, IISPV and Universitat Rovira i Virgili, Tarragona, Spain); Carlos Alonso-Villaverde, Gerard Aragonés, Antoni Castro, Pedro Pardo, Sandra Parra (Hospital Universitari de Sant Joan, IISPV and Universitat Rovira i Virgili, Reus, Spain); Pere Domingo, Àngels Fontanet, Mar Gutiérrez, Gràcia Mateo, Jessica Muñoz, Mª Antònia Sambeat (Hospital de la Santa Creu i Sant Pau and Universitat Autònoma de Barcelona, Barcelona, Spain); Victor Asensi, A-Hugo Montes, Eulalia Valle-Garay (Hospital Universitario Central de Asturias and Universidad de Oviedo, Oviedo, Spain).

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Address correspondence to:
Francesc Vidal
Infectious Diseases and AIDS Section
Department of Internal Medicine
Hospital Universitari de Tarragona Joan XXIII
IISPV
Universitat Rovira i Virgili
Mallafré Guasch, 4
43007 Tarragona, Spain

E-mail: fvidalmarsal.hj23.ics@gencat.cat

ESTUDIO 3

RESEACH ARTICLE

Effect of TNF-α genetic variants and CCR5Δ32 on the vulnerability to HIV-1 infection and disease progression in Caucasian Spaniards

Sergi Veloso, Montserrat Olona, Felipe García, Pere Domingo, Carlos Alonso-Villaverde, Montserrat Broch, Joaquim Peraire, Consuelo VIladés, Montserrat Plana, Enric PEdrol, Miguel López-Dupla, Carmen Aguilar, Mar Gutiérrez, Agathe León, Mariona Tasias, Josep Mª Gatell, Cristóbal Richart and Francesc Vidal.

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Effect of *TNF-α* genetic variants and *CCR5Δ32* on the vulnerability to HIV-1 infection and disease progression in Caucasian Spaniards

Sergi Veloso[1,2,3], Montserrat Olona[1,2,3], Felipe García[4], Pere Domingo[5,6], Carlos Alonso-Villaverde[2,3,7], Montserrat Broch[2,3,8], Joaquim Peraire[1,2,3], Consuelo Viladés[1,2,3], Montserrat Plana[4], Enric Pedrol[9], Miguel López-Dupla[1,2,3], Carmen Aguilar[1,2,3,8], Mar Gutiérrez[5], Agathe Leon[4], Mariona Tasias[9], Josep Ma Gatell[4,10], Cristóbal Richart[1,2,3,8] and Francesc Vidal*[1,2,3]

Abstract

Background: Tumor necrosis factor alpha (TNF-α) is thought to be involved in the various immunogenetic events that influence HIV-1 infection.

Methods: We aimed to determine whether carriage of the *TNF-α-238G>A, -308G>A* and *-863 C>A* gene promoter single nucleotide polymorphisms (SNP) and the *CCR5Δ32* variant allele influence the risk of HIV-1 infection and disease progression in Caucasian Spaniards. The study group consisted of 423 individuals. Of these, 239 were uninfected (36 heavily exposed but uninfected [EU] and 203 healthy controls [HC]) and 184 were HIV-1-infected (109 typical progressors [TP] and 75 long-term nonprogressors [LTNP] of over 16 years' duration). *TNF-α* SNP and the *CCR5Δ32* allele were assessed using PCR-RFLP and automatic sequencing analysis methods on white blood cell DNA. Genotype and allele frequencies were compared using the χ 2 test and the Fisher exact test. Haplotypes were compared by logistic regression analysis.

Results: The distribution of *TNF-α-238G>A, -308G>A* and *-863 C>A* genetic variants was non-significantly different in HIV-1-infected patients compared with uninfected individuals: *-238G>A*, p = 0.7 and p = 0.3; *-308G>A*, p = 0.05 and p = 0.07; *-863 C>A*, p = 0.7 and p = 0.4, for genotype and allele comparisons, respectively. Haplotype analyses, however, indicated that carriers of the haplotype H3 were significantly more common among uninfected subjects (p = 0.04). Among the infected patients, the distribution of the three *TNF-α* genetic variants assessed was non-significantly different between TP and LTNP: *-238G>A*, p = 0.35 and p = 0.7; *-308G>A*, p = 0.7 and p = 0.6: *-863 C>A*, p = 0.2 and p = 0.2, for genotype and allele comparisons, respectively. Haplotype analyses also indicated non-significant associations. Subanalyses in the LTNP subset indicated that the *TNF-α-238A* variant allele was significantly overrepresented in patients who spontaneously controlled plasma viremia compared with those who had a detectable plasma viral load (genotype comparisons, p = 0.02; allele comparisons, p = 0.03). The *CCR5Δ32* distribution was non-significantly different in HIV-1-infected patients with respect to the uninfected population (p = 0.15 and p = 0.2 for genotype and allele comparisons, respectively) and in LTNP vs TP (p = 0.4 and p = 0.5 for genotype and allele comparisons, respectively).

Conclusions: In our cohort of Caucasian Spaniards, *TNF-α* genetic variants could be involved in the vulnerability to HIV-1 infection. *TNF-α* genetic variants were unrelated to disease progression in infected subjects. The *-238G>A* SNP may modulate the control of viremia in LTNP. Carriage of the *CCR5Δ32* variant allele had no effect on the risk of infection and disease progression.

Background

TNF-α is a pleiotropic cytokine that acts as an immune and inflammatory mediator. It is synthesized mainly by macrophages and T-cells and most of its actions take place by binding to two cell receptors: TNF-α R1 and R2 [1]. Investigations suggest that TNF-α is involved in the pathogenesis of HIV-1 infection since it is overproduced by infected individuals [2-4].

* Correspondence: fvidalmarsal.hj23.ics@gencat.cat
[1] Hospital Universitari de Tarragona Joan XXIII, Tarragona, Spain
Full list of author information is available at the end of the article

Like many other proinflammatory cytokines, the production of TNF-α is at least partially genetically determined [5,6]. Several functional single nucleotide polymorphisms (SNP) have been identified within the *TNF-α* gene cluster [5-8], the most widely studied of which are a *G>A* transition at position *-238*, a *G>A* transition at position *-308*, and a *C>A* transition at position -*863* [9]. Several cohort studies have assessed the influence of *TNF-α* SNP on the immunogenetics of HIV-1 infection. The information available on how *TNF-α* genetic variants affect vulnerability to infection is inconsistent [10-14]: some studies have found no effect [11,13,14] while others have found a strong association [10,12]. Case-control studies have assessed the influence of *TNF-α* genetic variants on HIV-1 disease progression in infected patients [10-12,14-17]. While some of these studies have reported an association with progression [10-12,16,17], others have failed to find such an association [14,15]. A minority of untreated HIV-1-infected patients show an uncommon clinical form of infection characterized by a non-progressive disease and, sometimes, by self-limited viremia over the years. These patients are known as long-term nonprogressors (LTNP) [18] and elite controllers [19], respectively. Little data is available on the influence of *TNF-α* SNP on these uncommon clinical forms of HIV-1 infection [11,16].

In this study we have assessed the influence of *TNF-α* SNP on the vulnerability to HIV-1 infection and, in infected patients, on disease progression. For this purpose we analysed a cohort that we have already used for several immunogenetic studies on HIV-1 infection which contains a subset of repeatedly exposed but uninfected individuals and a carefully collected cohort of extreme long-term nonprogressors [20,21]. Given its modulating role in HIV-1-infection and disease progression reported elsewhere [22], the *CCR5Δ32* allele was also assessed.

Methods
Design and setting
This was a multicenter cross-sectional population association study. All subjects were recruited from a prospectively collected cohort of almost 6000 HIV-1-infected patients treated at the HIV outpatients' clinics of the participating hospitals, which are located in an epidemiological setting in which intravenous drug use is one of the main causes of HIV-1-infection.

Population
Two subsets of HIV-1-infected patients were studied: LTNP and typical progressors (TP). They were recruited between 2005 and 2007. Criteria for LTNP were: asymptomatic HIV-1 infection of over 16 years' duration; in the absence of any antiretroviral treatment, a stable CD4+ cell count persistently over 500 cells/μl; and a plasma

HIV-1 viral load repeatedly under 5000 copies/ml [18]. LTNP were further divided into two subgroups depending on whether the plasma viral load was detectable (LTNP-DVL) or persistently undetectable. LTNP in the latter subgroup were called elite controllers (LTNP-EC) [19]. Patients were categorized as TP if they fulfilled all the following criteria: a) the HIV-1 infection had progressed to the advanced disease (that is to say class C HIV-1 disease had appeared according to the 1993 Centers for Disease Control criteria [23]), b) the plasma HIV-1 viral load was over 35,000 copies/ml and, c) the CD4+ T-cell count decreased over time and was below 350 cells/μl at least once in the first 10 years of infection. Almost all of them were on antiretroviral therapy when they enrolled. For a few patients whose date of infection was not available, we assumed that it was the midpoint between the first positive and the last negative HIV-1 blood test [24,25]. We identified 75 patients within our cohort who fulfilled the LTNP criteria. They all agreed to participate in the study, and we recruited a randomly selected group of TP (n = 109) whose age (± 5 years) and gender were comparable with the LTNP. A group of 36 repeatedly exposed but uninfected individuals (EU) was also evaluated. They were part of a cohort of individuals that we have used in other studies and whose details and characteristics we have extensively described elsewhere [26,27]. For the control group we studied a sample of healthy subjects recruited from voluntary blood donors, whose age and gender were comparable with the patients. Table 1 shows details of the study population. All subjects in our study were white Spaniards. Immigrants from other countries, including those from other European countries, and their descendents were excluded. Informed consent was obtained from each participant. The project was approved by the local ethical research committees.

DNA and plasma samples
Blood samples with ethylene diamine tetra-acetic acid were obtained from an antecubital vein. Five mL of whole blood was used to determine the CD4+ T-cell count, and 500 μl was used to isolate DNA with a MagNa Pure LC Instrument (Roche Diagnostics, Basel, Switzerland). Plasma for determining HIV-1 viral load was obtained by centrifugation at 3500 g for 15 minutes at 4C°.

Laboratory methods
HIV-1 infection
This was diagnosed using an enzymoimmunoanalysis and confirmed by a Western-Blot test.
Plasma HIV-1 viral load
This was determined by the HIV Cobas Ampliprep CAP-CTMHIV-1 using the COBAS AMPLICOR system

Table 1: Demographic and clinical characteristics of the population analysed

Variable	Healthy controls (n = 203)	EU (n = 36)	HIV-1 TP# (n = 109)	HIV-1 LTNP# (n = 75)	*p* value
Male (%)	146 (72%)	22 (61%)	78 (71.5%)	50 (66.6%)	0.59*
Age (years) (mean ± SD)	44.2 ± 9.9	46.7 ± 9.2	41.8 ± 8.9	41.6 ± 7.1	0.39**
Duration of HIV-1 infection (mean ± SD)	-	-	6.2 ± 2.7	18.2 ± 1.6	< 0.0001 ##, ***
Time span between documented infection and the onset of antiretroviral treatment (mean ± SD)	-	-	4.8 ± 1.3	-	-
Plasma HIV-1 RNA (copies/ml) (median and range)	-	-	11,808 (<50->750,000)	267 (<50-4,600)	< 0.0001 ##, ****
CD4+ T-cell count (cells/μl) (median and range)	-	-	478 (6-1404)	772 (503-2080)	< 0.0001 ##,****
Exposure to HIV-1	-	22 (61%)	61 (56%)	47 (62.7%)	0.64*
Parenteral	-	14 (39%)	39 (35.8%)	22 (29.3%)	0.53*
Sexual	-	-	9 (8.2%)	6 (8%)	0.82*
Other					

• EU: Individuals repeatedly exposed to HIV-1 but uninfected
• HIV-1 TP: HIV-1-infected typical progressors
• HIV-1 LTNP: HIV-1-infected long-term non-progressors
• <50 was arbitrarily counted as 49
The studies made of HIV-1 TP and HIV-1 LTNP were cross-sectional and the viral load and CD4 cell counts are those of the date of selection. More than 90% of HIV-1 TP were under highly active antiretroviral therapy.
p value stems from the comparison between HIV-1 TP and HIV-1 LTNP.
* *p* value results of the χ^2 test;
** *p* value results of the ANOVA test
*** *p* value results of the Student T test
**** *p* value results of the Mann-Whitney U test

(Roche Diagnostics, Basel, Switzerland). The cutoff for undetectable viral load was 50 copies/μl.

Assessment of blood CD4+ T-cell count
Samples were analyzed in a flow cytometer FAC Scan (Becton Dickinson Immunocytometry Systems, San José, CA, USA). The data acquired were analyzed using the Multiset program.

TNF-α genotype
-238 TNF-α *genotype (rs 361525)*
This polymorphism consists of a GT A substitution at position -238 in the proximal promoter of the *TNF-α* gene. The primers used in PCR were: forward primer 5' AGAAGACCCCCCTCGGAACC3', modified in 3' for RFLP analysis with Msp I restriction endonuclease, and

reverse primer 5' ATCTGGAGGAAGCGGTAGTG 3'. A fragment of 152 bp was amplified at a final volume of 50 μL, with 3 mM of $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each primer and 1 unit of Taq polymerase. DNA was amplified for 35 cycles: 95° for 30 seconds, 55° for 30 seconds and 72° for 30 seconds. PCR products were digested with MspI and revealed a fragment of 152 bp for the A allele and two fragments of 133 and 19 bp for the G allele.

-308 TNF-α *genotype (rs 1800629)*
This polymorphism consists of a GT A substitution at position -308 in the proximal promoter of the *TNF-α* gene. The primers used in the PCR were 5'AGGCAATAGGTTTTGAGGGCCAT3' and 5'TCCT CCCTGCTCCGATTCCG3'. Amplification was performed at a final volume of 50 μl containing 3 mM $MgCl_2$,

0.5 mM of each nucleotide (Boehringer Mannheim™, Manheim, Germany), 0.2 μl of each oligonucleotide and 1 U of Taq polymerase (Gibco BRL). DNA was amplified for 35 cycles with denaturation at 94°C for 1 minute (min), annealing at 60°C for 1 min and extension at 72°C for 1 min. The first cycle was at 94°C for 3 min, at 60°C for 1 min and at 72°C for 1 min, followed by the 35 cycles and, finally, a cycle at 94°C for 1 min, at 60°C for 1 min and at 72°C for 5 min. The PCR products were digested at 37°C with NcoI for 24 hours, subjected to 2.5% agarose gel electrophoresis at 80 V and stained with ethidium bromide. The 107 bp band corresponded to the A allele and the set of 87 bp and 20 bp bands corresponded to the G allele.

-863 TNF-α *genotype (rs 1800630)*

This polymorphism consists of a CT A substitution at position -863 in the proximal promoter of the *TNF-α* gene. The primers used in the PCR were 5'GGCTCTGAGGAATGGGTTAC3' and 5'CTACAT GGCCCTGTCTTCGTTACG3'. Amplification was performed at a final volume of 25 μl containing 1 mM MgCl$_2$, 0.2 mM of each nucleotide (Boehringer Mannheim™, Manheim, Germany), 1.2 μl of each oligonucleotide and 0.5 U of Taq polymerase (Gibco BRL). DNA was amplified for 35 cycles with denaturation at 94°C for 30 seconds, annealing at 62.3°C for 1 min and extension at 72°C for 2 min. The first cycle was at 94°C for 3 min, followed by the 35 cycles and, finally, an extension at 72°C for 5 min. The amplified product was digested with Bsa AI restriction enzyme (New England Biolabs) at 37°C for 24 hours, electrophoresed on 2.5% agarose gel at 80 V and stained with ethidium bromide. The 126 pb band corresponded to the C wild-type allele and the set of 103 bp and 23 pb bands corresponded to the variant A allele.

CCR5Δ32 genotype

The PCR primers used were 5'GCTCTCTCCCAGGAA TCATC3' and antisense 5'TTCCCGAGTAGCAGAT GACC3' with annealing at 60°C. The Genbank accession number was AF031237.1. The PCR products were visualized on 2.5% agarose gel. The wild-type *CCR5* gene led to a 174 bp fragment and the *CCR5Δ32* variant resulted in a 142 bp fragment.

Statistical analysis

Descriptive data were expressed as the mean ± SD or median (range) for non-parametric distributions. The differences in levels between groups in continuous variables were compared using Student's t test, the Mann-Whitney U test, or ANOVA when necessary. To evaluate the association between HIV-1 TP and the different categories of uninfected subjects for the different genotypes, the odds ratio (OR) and 95% confidence interval (95%CI) were calculated. The Hardy-Weinberg equilibrium was assessed by the chi-square goodness-of-fit test. Genotype distribution and allele frequencies in the different groups were compared by the χ2 test or Fisher's exact test when necessary. To evaluate the association of *TNF-α* genetic variants and *CCR5Δ32* with the risk of HIV-1 infection, the differences in the distribution between HIV-1-infected, HC and EU were assessed. To evaluate the association of *TNF-α* genetic variants and *CCR5Δ32* with disease progression in infected subjects, we compared HIV-1-infected TP vs HIV-1-infected LTNP. In the latter subset, we also compared LTNP-DVL and LTNP-EC. Analyses were performed using the SPSS/PC+ statistical package (v. 12.0 for Windows; Chicago, Illinois, USA). Haplotype frequencies were estimated by the maximum-likelihood method. Haplotype frequencies were estimated by the PAC-likelihood method [28], which takes into account the similarity of haplotypes and the fact that linkage disequilibrium decays with distance. The distributions of the different haplotypes between groups were compared by logistic regression analysis. The commonest haplotype (which we arbitrarily called haplotype H1) was taken as a reference. Haplotype analyses were performed using the PHASEv2.1 software [29,30]. A *p* value < 0.05 was considered significant.

Results

Four hundred and twenty-three individuals were studied: 239 uninfected (203 healthy controls [HC] and 36 EU) and 184 HIV-1-infected. Of the infected individuals, most of whom had acquired HIV-1 through intravenous drug use, 109 were TP and 75 were LTNP. Of the LTNP, 20 were elite controllers. The age, gender and risk factors of acquiring HIV-1 infection in the TP and LTNP were not significantly different. Most TP (>90%) were receiving highly active antiretroviral therapy. Table 1 shows selected characteristics of the population studied. As expected, the duration of HIV-1 infection was significantly greater in LTNP than in TP. CD4+ T-cell counts were also greater and viral loads were lower.

TNF-α gene polymorphisms

Tables 2 and 3 show the genotype distribution and allele frequencies of the *TNF-α-238G>A, -308G>A* and *-863 C>A* gene promoter SNP and the *CCR5Δ32* allele for the control group and the different patient categories. The genotype distribution in control subjects, EU, HIV-1-infected, HIV-1 TP and HIV-1 LTNP fits the expected Hardy-Weinberg equilibrium for each *TNF-α* SNP.

The risk of infection showed no significant associations with the *TNF-α-238G>A* and *-863G>C* SNP; carriers of the *TNF-α-308A* genetic variant were overrepresented among uninfected individuals, but the differences did not reach statistical significance (genotype comparisons, p = 0.05; allele comparisons, p = 0.07) (Table 2).

Table 2: *TNF-α* and *CCR5Δ32* genotype and allele frequencies in HC, EU and HIV-1-infected for assessment of associations with the risk of HIV-1 infection.

Genotype and allele frequencies	HC (n = 203)	EU (n = 36)	HIV-1-infected (n = 184)	*p* value *
TNF-α-238 G>A				
GG	147 (87.5%)	29 (80.5%)	161 (87.5%)	
GA	20 (12%)	6 (16.7%)	22 (12%)	0.7
AA	1 (0.5%)	1 (2.8%)	1 (0.5%)	
GA+AA	21 (12.5%)	7 (19.5%)	23 (12.5%)	0.5
Variant allele *A*	22 (6.5%)	8 (11.1%)	24 (6.5%)	0.3
TNF-α-308G>A				
GG	122(70.9%)	24 (67%)	148 (80.4%)	
GA	42(24.4%)	12 (33%)	31 (16.8%)	0.05
AA	8 (4.7%)	0	5 (2.8%)	
GA+AA	50 (29.1%)	12 (33%)	36 (19.6%)	0.05
Variant allele *A*	58 (16.9%)	12 (16.7%)	41 (11.1%)	0.07
TNF-α-863 C>A				
CC	116 (69.9%)	27 (75%)	137 (74.4%)	
CA	44 (26.5%)	8 (22%)	45 (24.5%)	0.7
AA	6 (3.6%)	1 (3%)	2 (1.1%)	
CA+AA	50 (30.1%)	9 (25%)	47 (25.5%)	0.6
Variant allele *A*	56 (16.8%)	10 (14%)	49 (13.3%)	0.4
CCR5Δ32				
wt/wt	174 (87%)	31 (86.1%)	144 (78.3%)	
wt/Δ32	26 (13%)	5 (13.9%)	40 (21.7%)	0.15
Δ32/Δ32	0	0	0	
Δ32 allele	26 (6.5%)	5 (6.9)	40 (10.9%)	0.2

• HC: healthy controls
• EU: individuals repeatedly exposed to HIV-1 but uninfected
• wt indicates wild-type allele; *Δ32*, 32 bp deletion
• Genotype and allele numbers do not match the HC studied because DNA for *TNF-α* and *CCR5* genotyping was not available or could not be amplified in some people
* χ2 test or Fisher exact test when necessary. *p* value arises from the comparison between HC, EU and HIV-1-infected.

Table 3: *TNF-α* and *CCR5Δ32* genotype and allele frequencies in the different subsets of HIV-1-infected patients for assessment of associations with disease progression.

Genotype and allele frequencies	HIV-1 TP (n = 109)	HIV-1 LTNP (n = 75)	*p* value *	HIV-1 LNTP-DVL (n = 55)	HIV-1 LTNP-EC (n = 20)	*p* value **
TNF-α-238G>A						
GG	97 (90%)	64 (85.3%)		50 (90.9%)	14 (70%)	
GA	11 (10%)	11 (14.7%)	0.35	5 (9.1%)	6 (30%)	0.02
AA	1 (1%)	0		0	0	
GA+AA	12 (11%)	11 (14.7%)	0.6	5 (9.1%)	6 (30%)	0.02
Variant allele *A*	13 (6%)	11 (7.3%)	0.7	5 (4.5%)	6 (15%)	0.03
TNF-α-308 G>A						
GG	86 (78.9%)	62 (82.7%)		46 (83.6%)	16 (80%)	
GA	20 (18.3%)	11 (14.7%)	0.7	8 (14.5%)	3 (15%)	0.7
AA	3 (2.8%)	2 (2.6%)		1 (1.9%)	1 (5%)	
GA+AA	23 (21.1%)	13 (17.3%)	0.5	9 (16.4%)	4 (20%)	0.7
Variant allele *A*	26 (11.9%)	15 (10%)	0.6	10 (9.1%)	5 (12.5%)	0.7
TNF-α-863 C>A						
CC	84 (77.1%)	53 (70.6%)		37 (67.3%)	16 (80%)	
CA	25 (22.9%)	20 (26.6%)	0.2	17 (30.9%)	3 (15%)	0.3
AA	0	2 (2.8%)		1 (1.8%)	1 (5%)	
CA+AA	25 (22.9%)	22 (29.3%)	0.4	18 (32.7%)	4 (20%)	0.3
Variant allele *A*	25 (11.5%)	24 (16%)	0.2	19 (17.3%)	5 (12.5%)	0.4
CCR5Δ32						
wt/wt	88 (80.7%)	57 (76%)		39 (70.9%)	18 (90%)	
wt/Δ32	21 (19.3%)	18 (24%)	0.4	16 (29.1%)	2 (10%)	0.1
Δ32/Δ32	0	0		0	0	
Variant allele *Δ32*	21 (9.6%)	18 (12%)	0.5	16 (14.5%)	2 (5%)	0.1

• HIV-1 TP: HIV-1-infected typical progressors
• HIV-1 LTNP: HIV-1-infected long-term nonprogressors
• HIV-1 LTNP-DVL: HIV-1-infected long-term nonprogressors with detectable plasma viral load
• HIV-1 LTNP-EC: HIV-1-infected long-term nonprogressors with undetectable plasma viral load
• wt indicates wild-type allele; *Δ32*, 32 bp deletion
* χ2 test or Fisher exact test when necessary. *p* value arises from the comparison between HIV-1 TP and HIV-1 LTNP.
** χ2 test or Fisher exact test when necessary. *p* value arises from the comparison between HIV-1 LTNP-DVL and HIV-1 LTNP-EC.

Both genotype and allele analyses indicated that there were no differences in the distribution of the genetic variants between LTNP and TP (Table 3). When we analysed the LTNP subset of patients and compared the elite controllers with those with detectable viral loads, we found that the *TNF-α-238A* genetic variant was significantly overrepresented in elite controllers: p = 0.02 for genotype analyses and p = 0.03 for allele analyses (Table 3). The results obtained in the full cohort for all these analyses were maintained when the individuals carrying the *CCR5Δ32* variant allele were excluded.

TNF-α haplotype analyses indicated that there was a significant association between carriage of the haplotype 3 and the risk of infection (p = 0.04) (Table 4). No significant associations were observed with disease progression in infected individuals (Table 5). It should be noted that the EU subset could not be included in the analyses because of the low number of individuals in our cohort.

CCR5 Δ32
There were no significant differences in the prevalence of the *CCR5Δ32* genetic variant allele among the different categories of patients assessed (Tables 2 and 3). *CCR5Δ32* homozygosity was not detected.

Discussion
In this cohort of Caucasian Spaniards, we found that *TNF-α* gene polymorphism may be involved in vulnerability to HIV-1 infection. In infected patients, none of the *TNF-α* genetic variants assessed influences disease progression, but the *TNF-α-238G>A* SNP may modulate viremia control in LTNP. Carriage of the *CCR5Δ32* vari-

ant allele does not influence the risk of infection or disease progression.

To explain the variable interindividual vulnerability to HIV-1 infection, we sought a genetically-driven host susceptibility. Several candidate genes were checked, one of which was *TNF-α* because of its robust role in initial response to infection influences both innate and acquired immunity [1,3,31,32]. Our data indicate that none of the individual polymorphisms assessed influence vulnerability to HIV-1 infection, but haplotype analyses suggest that the combination of some genetic variants within the *TNF-α* gene may modulate the risk of infection. These results agree with some reports that showed an association between *TNF-α* SNP and the risk of infection [10,12]; other investigations, however, observed no significant associations [11,13-15]. The discrepancy between our findings and those reported previously may be because of the low number of patients assessed in some studies [15], which often provides unstable data [33], or because the different ethnicities of the people assessed in others [13,14] may have led to genuine population differences [34].

Other reports have investigated the involvement of *TNF-α* genetic variants in a variety of immunogenetic events that affect HIV-1 disease progression. Some studies have related the risk of progression [10-12,16,17] and/ or of developing a diversity of AIDS-related events [35-41] with carriage of some *TNF-α* genetic variants while others, like ours, have failed to find any significant association [14,15]. In this respect, our study suggests that there are no significant associations between *TNF-α* genetic variants and disease progression. To assess this issue we used a cohort of TP as controls and a subset of LTNP of

Table 4: *TNF-α* haplotypes and risk of HIV-1-infection.

Haplotypes	*TNF-α* SNP			n (estimated frequencies in %)			OR	OR 95CI%	*p* value *
	-238 G>A	*-308 G>A*	*-863 G>A*	Total (n = 373)	Uninfected (n = 189)[1]	HIV-1-Infected (n = 184)			
H1	G	G	C	246 (65.98%)	115 (61.45%)	131 (70.73%)	1.00		
H2	G	G	A	54 (14.46%)	31 (16.66%)	23 (12.51%)	1.47	0.95 - 2.30	0.09
H3	G	A	C	48 (13.07%)	29 (15.36%)	19 (10.45%)	1.65	1.04 - 2.64	0.04
H4	A	G	C	21 (5.45%)	10 (5.13%)	11 (5.99%)	1.03	0.52 - 2.04	0.94
rare	*	*	*	5 (1.04%)	-	-	1.46	0.23 - 9.47	0.69

Note: The exposed uninfected subset of patients could not be included in the analysis because of the low number of individuals available.
[1]Of 203 healthy uninfected controls individuals, only 189 had the three *TNF-α* SNPs assessed.
* *p* value for comparisons of each haplotype with a frequency greater than 1% with the most common haplotype (H1)

Table 5: *TNF-α* haplotypes and disease progression in HIV-1-infected patients.

Haplotypes	*TNF-α* SNP			n (estimated frequencies in %)			OR	OR 95CI%	*p* value *
	-238 G>A	*-308 G>A*	*-863 G>A*	Total (n = 184)	HIV-1 TP (n = 109)	HIV-1 LTNP (n = 75)			
H1	G	G	C	131 (70.73%)	80 (73.44%)	51 (66.70%)	1.00		
H2	G	G	A	23 (12.51%)	12 (9.90%)	11 (16.04%)	1.83	0.92 - 3.64	0.09
H3	G	A	C	19 (10.45%)	12 (9.83%)	7 (9.95%)	1.20	0.60 - 2.41	0.61
H4	A	G	C	11 (5.99%)	5 (4.25%)	6 (7.30%)	1.84	0.68 - 4.95	0.23
rare	*	*	*	0 (0.32%)	-	-	1.00	-	1.00

* *p* value for comparisons of each haplotype with a frequency greater than 1% with the most common haplotype (H1)
HIV-1 TP: HIV-1-infected typical progressors
HIV-1 LTNP: HIV-1-infected long-term non-progressors

over 16 years' duration as cases. This extreme LTNP phenotype was expressly chosen to prevent groups from superposing. Of particular note, however, was that within the LTNP group we showed a significant association between carriage of the *TNF-α-238A* variant allele and the spontaneous "elite controller" phenotype. This replicates what was reported in an early study [16] but, since this finding is supported by a very small number of patients it should be further replicated in larger series of this uncommon subset of HIV-1-infected patients.

For *CCR5Δ32*, we found that there is no signal for non progression. This is in agreement with *CCR5Δ32* having a primary effect against rapid progression as pointed out by the GRIV study [42,43]. We acknowledge, however, that in order to see the effect of *CCR5Δ32*, the use of a larger cohort and/or a Kaplan-Meier plot of patients' evolution since seroconversion under various endpoints could have been useful.

Our study has some limitations. First, the cross sectional nature of the design provides associations, not causality. Second, the comparison of the LTNP with the TP subsets is rather limited since it involves long-term non progression and not simple progression, and additional analyses with Kaplan-Meier curves (with various endpoints such as death or AIDS 1993 criteria) could lead to different results. Finally, some of the subsets assessed were small, particularly the exposed uninfected and elite controllers. This means that our analyses of these subsets may be too underpowered to detect other significant associations.

Conclusions

In summary, in a cohort of Caucasian Spaniards, polymorphism within the *TNF-α* gene may be associated with vulnerability to HIV-1 infection. In infected patients, none of the *TNF-α* genetic variants assessed influences disease progression but the *TNF-α-238G>A* SNP may modulate the elite controller status. The *CCR5Δ32* variant allele influences neither the risk of infection nor disease progression.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
SV, FG, PD, CAV, EP, JMG, CR and FV designed the study. SV, JP, CV, MG, AL and MT collected and analysed the data. MB, MP and CA performed the genetic studies. MO and MLD performed the statistical analyses. SV, MO, FG, PD, CAV, EP, JMG, CR and FV drafted the manuscript. All authors have read and approved the manuscript.

Author Details
[1]Hospital Universitari de Tarragona Joan XXIII, Tarragona, Spain, [2]IISPV, Tarragona, Spain, [3]Universitat Rovira i Virgili, Tarragona, Spain, [4]Hospital Clínic, Barcelona, Spain, [5]Hospital de la Santa Creu i Sant Pau, Barcelona, Spain, [6]Universitat Autònoma de Barcelona, Barcelona, Spain, [7]Hospital Universitari de Sant Joan, Reus, Spain, [8]CIBER Fisiopatologia de la Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III, Madrid, Spain, [9]Hospital de Sant Pau i Santa Tecla, Tarragona, Spain and [10]Universitat de Barcelona, Barcelona, Spain

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E. DISCUSION

ESTUDIO 1: Tumour necrosis factor alpha in fat redistribution syndromes associated with combination antiretroviral therapy in HIV-1 infected patients: potential role in subcutaneous adipocyte apoptosis

Uno de los hallazgos histopatológicos en la LD es la existencia de una apoptosis adipocitaria incrementada en el TAS. El TNF-α es un potente inductor de la apoptosis, del fenómeno inflamatorio y se ha descrito su disregulación en pacientes infectados por el VIH que realizan TARGA y que presentan LD.

Estudiar esa posible relación es el objetivo del presente estudio. Los pacientes se dividieron en 3 grupos: Pacientes infectados por el VIH (con y sin LD) y un grupo control sano. Para evitar sesgos, la LD debía ser moderada-severa en el grupo de LD y los pacientes sin LD llevaban suficiente tiempo con TARGA para poder descartar de manera razonable la aparición de LD en el seguimiento.

Los resultados más destacados del estudio son los siguientes:

1. Mayor grado de apoptosis adipocitaria en el TAS en pacientes con LD que en pacientes sin LD y controles (p= 0,0001 y p< 0,0001 respectivamente)

2. Correlación significativa entre los niveles de sTNFR1 Y R2 y el grado de apoptosis del TAS (p< 0,0001 para ambos receptores)

3. En el cultivo celular, el TARGA ejerce un efecto pro-apoptótico significativo que se potencia con la adición de TNF-α. Éste último presenta, además, un efecto apoptótico independiente de la presencia de ARV.

Los pacientes con infección por VIH sin tratamiento presentan niveles elevados de TNF-α. Al iniciar el TARGA, estos niveles descienden pero sin llegarse a normalizar. Esto es debido, entre otras causas, a una producción aumentada de TNF-α en el TAS producida por las proteínas del propio VIH, el TARGA y un acumulo progresivo de células T productoras de TNF-α por disminución de la apoptosis.

Un hallazgo anatomo-patológico constante en las biopsias de TAS en pacientes con LD es la presencia constante de lipogranulomas, dato que sugiere inflamación local producida, entre otros, por exceso de TNF-α. El aumento de mRNA del TNF-α y su secreción en cultivos adipocitarios en pacientes con LD así parece confirmarlo.

Todas estas observaciones sugieren la presencia de un ambiente proinflamatorio con alteración del sistema del TNF-α en el TAS provocado por el propio VIH y el TARGA que, en pacientes con cierta predisposición genética puede provocar mayor apoptosis en TAS y la aparición de LD clínica.

Las principales limitaciones del estudio vienen determinadas por su naturaleza transversal (cross-sectional study) que dificulta inferir la causalidad en las asociaciones descritas. Otra posible limitación es la obtención de una única determinación de citoquinas en plasma, dada su variabilidad. Para evitar al máximo esto, la determinación fue realizada a pacientes con estabilidad clínica, sin patología inflamatoria / infecciosa concomitante y sin toma de fármacos que pudieran alterar la respuesta inflamatoria como corticoides.

ESTUDIO 2: No relationship between TNF-α genetic variants and combination antiretroviral therapy-related lipodystrophy syndrome in HIV type 1-infected patients: A case-control study and a meta-analysis.

La producción del TNF-α está, al menos parcialmente, determinada genéticamente. Se conocen diferentes polimorfismos genéticos que influyen en la secreción del mismo. Los más estudiados han sido los SNP -238G>A, -308C>A y -863C>A. A nivel de la LD asociada al VIH existen resultados contradictorios en cuanto a su influencia en el desarrollo de la misma.

En este segundo trabajo, un estudio transversal de asociación, se evaluaron 489 pacientes, 286 infectados por el VIH y 203 controles sanos y se complementó con la realización de un metaanálisis que incluía nuestros resultados y los obtenidos por otros grupos de estudio.

Los principales resultados del estudio transversal fueron los siguientes:

1. Ninguno de los polimorfismos genéticos del TNF-α influyen en la susceptibilidad a desarrollar LD en nuestra cohorte.

2. Existe un aumento de los niveles sistémicos de TNF-α en pacientes infectados en tratamiento con TARGA independientemente de la presencia o no de LD.

3. Relación de la LD con enfermedad por VIH avanzada, mayor recuperación inmunológica y el tiempo de TARGA, particularmente la exposición a d4T.

Los resultados del metanálisis indican la ausencia de relación entre los polimorfismos estudiados (-238G>A y -308G>A) y la aparición de LD. Son datos obtenidos de 284 pacientes con LD y 338 sin LD, hecho que da solidez a los resultados en comparación con los estudios previos con cohortes más pequeñas.

Los primeros trabajos publicados en relación al efecto de los polimorfismos del TNF-α en la aparición de LD (Maher[36] y Nolan[172]) mostraban un efecto modulador del -238G>A. Posteriores estudios de 2 cohortes (suiza y española) no encuentran dicha asociación, coincidiendo con nuestros resultados. El escaso número de pacientes estudiado en los primeros trabajos hace que la falta de asociación encontrada en los estudios posteriores y el nuestro tenga mayor robustez estadística al trabajar con mayor número de pacientes.

Sin embargo, estos resultados no invalidan la relación encontrada en el grupo de Nolan entre el ser portador del alelo 238A y la aparición más rápida de LD, ya que al ser un estudio longitudinal aporta información adicional.

Otro resultado destacable es la elevación sistémica de los niveles de TNF-α, tanto en pacientes con LD como sin ella. Este dato sugiere que el efecto del TNF-α en el TAS pueda estar relacionado más con un efecto local en el mismo que por una activación sistémica del sistema del TNF-α.

Las 2 limitaciones fundamentales de nuestro estudio son, primero, su naturaleza transversal, que dificulta el inferir causalidad en las asociaciones descritas y, segundo, la ausencia de una definición objetiva de LD, que puede hacer que haya casos de falsos negativos (pacientes con LD leve incluidos en el grupo de no LD). Para evitar esto, los pacientes del grupo de LD la presentan en grado moderado-severo y el grupo de no LD ha sido seguido durante un periodo de tiempo suficientemente largo sin haberse encontrado evolución a LD en estos pacientes.

En relación con el metanálisis, la principal limitación es la posible variabilidad en la definición de LD para incluir al paciente en un grupo u otro en los diferentes estudios, pudiendo dar lugar a grupos algo heterogéneos.

ESTUDIO 3: Effect of TNF-a genetic variants and CCR5Δ32 on the vulnerability to HIV-1 infection and disease progression in Caucasian Spaniards

En este tercer trabajo, se intenta determinar el papel de los diferentes polimorfismos del TNF-a (-238, -308 y -863) y del co-receptor CCR5Δ32 en los mecanismos inmunopatogénicos que determinan la vulnerabilidad y la progresión de la infección por VIH. Para ello, se estudiaron 423 pacientes, 184 pacientes infectados por el VIH (con progresión típica, no progresadores y controladores de élite) y 239 no infectados

Respecto al TNF-a, los resultados más destacados son los siguientes:

<u>Vulnerabilidad a la infección:</u>

1. No encontramos asociaciones significativas entre los polimorfismos del TNF-a y el riesgo de infección. La variante genética -308A está más representada en los pacientes no infectados pero sin significancia estadística

2. El haplotipo 3 (-238G, -308A y -863C) es más común en los pacientes no infectados con significancia estadística (p: 0.04).

<u>Progresión de la enfermedad:</u>

1. No existen diferencias entre los polimorfismos del TNF-a y la progresión de la enfermedad.

2. En el grupo de LTNP, los controladores de élite (CV negativas) se asocian con ser portadores de la variante alélica -238A

Todos estos resultados se mantienen incluso al excluir a los pacientes con la variante alélica de CCR5Δ32.

Respecto a CCR5, los resultados muestran que ser portador de la variante alélica CCR5Δ32 no influye en el riesgo de infección ni en el de progresión de la enfermedad.

El TNF-α es un buen gen candidato para estudiar la posible variabilidad genética en la vulnerabilidad a la infección por VIH debido a su importante papel en la respuesta del organismo a las infecciones. Los resultados de diferentes estudios son dispares. Nuestros datos (correlación haplotípica y riesgo de infección) coinciden con los de algunos autores al encontrar relación de los polimorfismos de TNF-α y el riesgo de infección. Sin embargo, otros autores no encuentran asociaciones significativas. Una posible explicación para esta disparidad es el escaso número de pacientes estudiados y las diferencias étnicas de la población estudiada. En nuestro caso, si bien no encontramos relación si estudiamos los polimorfismos de forma individual, el análisis haplotípico sugiere que la combinación de algunas variantes genéticas puede modular el riesgo de infección

Encontramos la misma disparidad de resultados cuando analizamos la variabilidad genética y la progresión de la enfermedad. Nosotros no hemos encontrado asociación entre los diferentes polimorfismos estudiados y la mayor / menor progresión de la infección. Para nuestra cohorte, utilizamos pacientes progresadores habituales como control y un grupo de LTNP con un seguimiento medio de 16 años para evitar posible solapamiento entre los grupos. Es de destacar, la asociación encontrada con la variante 238A en los pacientes controladores de élite ya sugerida en un estudio previo[194].

La ausencia de relación entre ser portador de la variante alélica CCR5Δ32 y la vulnerabilidad / progresión de la enfermedad en nuestra cohorte puede ser debida a que, a diferencia de otras cohortes que si la encuentran, la nuestra está formada mayoritariamente por pacientes que se infectaron por vía parenteral y por esta vía de infección, el papel protector del CCR5Δ32 puede no ser tan importante ya que también puede utilizarse la vía del CXCR4, a diferencia de la infección por vía sexual.

Respecto a las limitaciones del estudio, son similares a los previos en relación a su naturaleza transversal y al escaso número de pacientes en algunos de los grupos que puede afectar a la potencia estadística a la hora de realizar asociaciones.

F. CONCLUSIONES

1. TNF-α Y LIPODISTROFIA ASOCIADA AL VIH

1. Existe un aumento de los niveles sistémicos de TNF-α en pacientes infectados en tratamiento con TARGA independientemente de la presencia o no de LD.

2. Los pacientes con LD presentan mayor apoptosis adipocitaria que los pacientes sin LD y ésta se relaciona significativamente con los niveles de sTNF-α R1 y R2.

3. No encontramos relación entre los diferentes polimorfismos genéticos del TNF-α y el riesgo a desarrollar LD en los pacientes infectados por VIH y en TARGA.

4. "In vitro" (cultivo de adipocitos), el TNF-α es un inductor independiente de apoptosis y este efecto se potencia al añadir el TARGA en el cultivo.

5. Estos datos parecen sugerir un efecto inflamatorio a nivel local en el TAS sin relación con la actividad sistémica del TNF-α.

2. TNF-α E HISTORIA NATURAL DE LA INFECCION POR VIH

1. El análisis haplotípico muestra que el ser portador del haplotipo H3, (-238G, -308A, -863C) puede ser factor protector para la vulnerabilidad de la infección por VIH.

2. No hay relación entre los polimorfismos y la progresión de la enfermedad. Sin embargo, los controladores de élite presentan con mayor frecuencia la variante alélica -238G>A.

3. No hemos encontrado relación entre ser portador de la variante alélica CCR5Δ32 ni con la vulnerabilidad a la infección ni con la progresión de la misma.

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