UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA
Departamento de Pediatría

TESIS DOCTORAL

Arteriosclerosis carotidea en niños y adolescentes infectados por el VIH: factores fisiopatológicos implicados.

Atherosclerosis in HIV infected children and adolescents : related physiopatholgycal factors

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Directores

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María Ángeles Muñoz Fernández

Madrid, 2015

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MEMORIA PARA LA OBTENCIÓN DEL TÍTULO DE DOCTOR

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Arteriosclerosis Carotidea en niños y adolescentes infectados por el VIH: factores fisiopatológicos implicados

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“The more you learn in this business, the less you know”.
Omar Little

“Cuando creíamos que teníamos todas las respuestas, de pronto, cambiaron todas las preguntas”.
Mario Benedetti

“Al fin y al cabo, somos lo que hacemos para cambiar lo que somos”
Eduardo Galeano

A mi madre

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ABREVIATURAS

ABREVIATURAS EN ESPAÑOL

µl Microlitros

Ac Anticuerpo

ADN Ácido desoxirribonucleico

AHA American Heart Association

ARN Ácido ribonucleico

ARV Antirretroviral(es)

CD14 CD14 soluble

CD40L CD40 ligando

céls Células

CMV Citomegalovirus

CoRISpe Cohorte de la Red de Investigación en SIDA pediátrico

CV Carga viral del VIH-1

FI Factor de impacto

GIM Grosor íntima-medio carotideo

IC Intervalo de confianza

IMC Indice de masa corporal

IL-6 Interleuquina 6

IP Inhibidores de la proteasa

ITIAN Inhibidores de la transcriptasa inversa análogos de nucleós(t)ido

ITINAN Inhibidores de la transcriptasa inversa no análogos de núcleos(t)ido

log Logaritmo decimal

LPS Lipopolisacárido bacteriano

MALT Tejido linfoide asociado a mucosas

MCP-1 proteína quimioatrayente de monocitos

ml Mililitros

MPO Mieloperoxidasa

NS Estadísticamente no significativo

OMS Organización Mundial de la Salud

OR Odds ratio

PCR proteína C reactiva

RCV Riesgo cardiovascular

RIQ Rango intercuartílico

RIS Red de Investigación en SIDA

SIDA Síndrome de la inmunodeficiencia adquirida

sVCAM Molécula de adhesión vascular

TAR Tratamiento antirretroviral

TNF-α factor de Necrosis Tumoral Alfa

VHB Virus de la hepatitis B

VHC Virus de la hepatitis C

VIH Virus de la inmunodeficiencia humana

ABREVIATURAS EN INGLÉS

AIDS Acquired Immune Deficiency Syndrome

ART Antiretroviral Treatment

CD40L CD40 ligand

CMV Citomegalovirus

CVD cardiovascular disease

GALT Gut Associated Lymphoid Tissue

HIV Human Immunodeficiency Virus

IL-6 Interleukin 6

IMT carotid intima-media thickness

MCP-1 Monocyte chemo attractant protein 1

MPO Myeloperoxidase

sVCAM soluble vascular cell adhesion molecule

sCD14 soluble CD14

TNF-α Tumor Necrosis Factor Alpha

La presente tesis doctoral, de acuerdo con el informe correspondiente autorizado por los directores de tesis, y en cumplimiento con la normativa aprobada por el Órgano Responsable del Programa de Doctorado, se presenta como un compendio de cuatro publicaciones. Dos ya publicadas, la tercera aceptada para su publicación y la cuarta en segunda revisión en el momento de redacción de esta Memoria. Las referencias completas de los artículos que constituyen el cuerpo de la tesis son los siguientes:

1- Sainz T, Alvarez-Fuente M, Navarro ML, Díaz L, Rojo P, Blázquez D, Isabel de José M, Ramos JT, Serrano-Villar S, Martínez J, Medrano C, Muñoz-Fernández MA, Mellado MJ. **Subclinical atherosclerosis and markers of immune activation in HIV-infected children and adolescents: The CaroVIH Study.** J Acquir Immune Defic Syndr. 2014 Jan 1;65(1):42-9. FI: 4.43 (1er cuartil, Enfermedades Infecciosas).

2- Sainz T, Diaz L, Navarro ML, Rojo P, Blázquez D, Ramos JT, de José MI, Alvarez-Fuente M, Serrano-Villar S, Mellado MJ, Muñoz-Fernández MA. **Cardiovascular Biomarkers in Vertically HIV-infected Children Without Metabolic Abnormalities**. Aceptado para su publicación en Atherosclerosis en Enero de 2014. FI: 3.9 (1er cuartil, Enfermedad Vascular)

3- Sainz T, Serrano-Villar S, Díaz L, González Tomé MI, Gurbindo MD, de José MI, Mellado MJ, Ramos JT, Zamora J, Moreno S, Muñoz-Fernández MA. **The CD4/CD8 ratio as a marker T-cell activation, senescence and activation/exhaustion in treated HIV-infected children and young adults**. AIDS. 2013 Jun 1;27 (9):1513-6. FI: 6.245 (1er decil, Enfermedades Infecciosas).

4- Sainz T, Ortega-Hernández A, Serrano-Villar S, Navarro ML, Rojo P, Ramos JT, Mellado MJ, Diaz L, Alvarez-Fuente M, Estrada V, Gomez-Garre D, Muñoz-Fernández MA. **Functionally defective high density lipoproteins (HDL) are related to heightened T-cell activation in Vertically HIV-infected Adolescents**. En revisión en J Acquir Immune Defic Syndr desde Noviembre de 2013. FI: 4.43 (1er cuartil, Enfermedades Infecciosas).

Así mismo, se ha considerado oportuno mencionar que los resultados expuestos en esta Memoria han sido presentados en forma de Conferencia oral en los siguientes foros científicos, nacionales e internacionales. A continuación se enumeran las presentaciones orales de los trabajos integrados en esta Memoria y los premios obtenidos.

1. Conferencia de cierre del VI Congreso de la Sociedad Española de Infectología Pediátrica, Bilbao, Marzo de 2012. Premio a la Mejor Comunicación oral.
2. Participación en la Mesa Redonda sobre complicaciones del tratamiento antirretroviral en los niños y adolescentes infectados por el VIH. 29th Conference on Retroviruses and Opportunistic Infections, CROI, Seattle, March 2012. Premio jóvenes investigadores (Young Investigator Award).
3. Conferencia invitada en la Reunión del Grupo de Estudio de Alteraciones Metabólicas asociadas al VIH (GEAM), Marbella, Mayo de 2012.
4. Comunicación oral en el 61 º Congreso Nacional de la Sociedad Española de Pediatría, Granada, Junio de 2012. Premio a la Mejor Comunicación oral.

Por último, se ha considerado oportuno incluir en el Anexo I todos los artículos que han constituido la base formativa del doctorando y en los cuales ha participado como coautor durante el periodo de realización de la tesis, y que se enumeran a continuación:

1- Sainz T, Alvarez-Fuente M, Navarro ML, Díaz L, Rojo P, Blázquez D, Isabel de José M, Ramos JT, Serrano-Villar S, Martínez J, Medrano C, Muñoz-Fernández MA, Mellado MJ. **Cardiac Function in HIV-Infected Children and Adolescents in the ERA of HIghltly Active Antiretroviral Therapy**. En revisión en el Peditr Infec Diseas desde Octubre de 2013.

2- Sergio Serrano-Villar, Talia Sainz, Sulggi A.Lee, Peter W. Hunt, Elizabeth Sinclair, Barbara L. Shacklett, April L. Ferre4, Timothy L. Hayes, Ma Somsouk, Priscilla Y. Hsue, Mark Van Natta, Curtis L. Meinert, Michael M. Lederman, Hiroyu Hatano, Vivek Jain, Yong Huang, Frederick M. Hecht, Jeffrey N. Martin, Joseph M. McCune, Santiago Moreno and Steven G. Deeks. **HIV-infected Individuals With Low**

CD4/CD8 Ratio Despite Effective Antiretroviral Therapy Exhibit Altered T-cell Subsets, Heightened CD8+ T-cell Activation, and Increased Risk of Non-AIDS Morbidity/Mortality. En revisión en PLOS Pathogens desde Octubre de 2013.

3- Serrano-Villar S, Moreno S, Fuentes-Ferrer M, Sánchez-Marcos C, Avila M, Sainz T, de Villar N, Fernández-Cruz A, Estrada V. **The CD4:CD8 ratio is associated with markers of age-associated disease in virally suppressed HIV-infected patients with immunological recovery.** HIV Med. 2014 Jan;15(1):40-9. IF: 3.75, (2º cuartil, Infectious Diseases)

4- Serrano-Villar S, Estrada V, Gómez-Garre D, Avila M, Fuentes-Ferrer M, San Román J, Soriano V, Sánchez-Parra C, Sainz T, Fernández-Cruz A. **Diagnosis of subclinical atherosclerosis in HIV-infected patients: higher accuracy of the D:A:D risk equation over Framingham and SCORE algorithms.** Eur J Prev Cardiol. 2012 Jun 29. [Epub ahead of print]

5- Serrano-Villar S, Estrada V, Gómez-Garre D, Avila M, Fuentes-Ferrer M, Sánchez-Parra C, Sainz T, de Carranza M, Fernández-Cruz A. **Clinical factors and biomarkers associated with subclinical atherosclerosis in the human immunodeficiency virus infection**. Med Clin (Barc). 2012 Sep 8;139(6):231-7. Epub 2012 Mar 21 Spanish. PMID: 22440139

6- Serrano-Villar S, Estrada V, Gómez-Garre D, Avila M, Fuentes-Ferrer M, Sánchez-Parra C, Sainz T, Patiño R, Fernández-Cruz A. **Incipient Renal Impairment as a Predictor of Subclinical Atherosclerosis in HIV-Infected Patients.** J Acquir Immune Defic Syndr. 2012 Feb 1;59(2):141-148.

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RESUMEN

RESUMEN

La Organización Mundial de la Salud estima en 34 millones el número de personas vivían con el VIH a finales de 2012. De ellos, 2,5 millones eran niños[1]. Durante el año 2012, 350.000 niños se infectaron por el VIH y 250.000 murieron por causas relacionadas con el SIDA. El acceso al tratamiento antirretroviral (TAR) ha cambiado drásticamente la esperanza de vida de los niños infectados por el VIH; principalmente debido a la reducción en la incidencia de infecciones oportunistas, encefalopatía, morbilidad y mortalidad por todas las causas. Sin embargo, los datos muestran que la esperanza de vida de las personas infectadas por el VIH sigue siendo más baja que la de población general[2], principalmente debido a un aumento en la incidencia de enfermedades no asociadas a SIDA, un conjunto de patologías generalmente asociadas con el envejecimiento prematuro, y que incluye la enfermedad cardiovascular, insuficiencia renal, enfermedad hepática, osteoporosis y tumores no asociados a SIDA[3].

Entre otras patologías asociadas al envejecimiento prematuro, se ha descrito en pacientes infectados por el VIH un aumento de enfermedad arteriosclerótica y a una edad relativamente joven. Dado que la edad media de las personas infectadas por el VIH está aumentando progresivamente, es probable que la enfermedad cardiovascular adquiera una mayor importancia como causa de mortalidad en los próximos años[4]. A pesar de ser foco de intensa investigación, las causas del aumento del riesgo cardiovascular en esta población no se conocen con exactitud, debido probablemente a que se trata de un proceso multifactorial. Un estado pro-inflamatorio y pro-oxidativo secundario a la infección en sí, así como a los fenómenos de translocación bacteriana y activación inmune, junto con los efectos indeseables de la terapia antirretroviral y factores de enfermedad cardiovascular clásicos se han sugerido como los principales promotores del proceso aterogénico asociado a la infección por el VIH[5]. El desarrollo de la placa de ateroma comienza en edades tempranas de la vida[6], y el estudio de este proceso en fase subclínica en niños y adolescentes ofrece la oportunidad de aclarar la función específica de los diferentes factores en ausencia de factores de riesgo cardiovascular clásicos.

Actualmente existen diferentes métodos no invasivos que permiten evaluar el desarrollo de enfermedad cardiovascular, tales como la medición de la rigidez arterial, la

función endotelial o el engrosamiento de las capas íntima y media arterial (grosor íntima-media - GIM), que pueden verse complementados por la determinación de múltiples biomarcadores. Estudios previos utilizando el GIM como marcados subrogado han mostrado un aumento del riesgo cardiovascular en niños infectados por el VIH[7,8]. Otros estudios han descrito en esta población niveles más altos de biomarcadores inflamatorios, como la proteína C reactiva (PCR), aunque los resultados de los diferentes estudios no son concluyentes[7,9]. Hasta la fecha, ningún estudio se ha propuesto analizar en niños y adolescentes la existencia de una posible correlación entre la expresión de marcadores de activación /senescencia en células T y la presencia de fenómenos clínicos característicos del envejecimiento acelerado, tales como la arteriosclerosis. Sin embargo, se ha descrito una mayor frecuencia de estos dos fenotipos de células T en niños infectados por el VIH[10,11] .

En la actualidad, la esperanza de vida de los niños infectados por el VIH y en TAR se ha incrementado notablemente, y sin embargo aún no es comparable a la de la población general. Disponer de herramientas que permitan identificar a los sujetos con mayor riesgo de presentar complicaciones asociadas resulta prioritario especialmente en la población única de pacientes con infección de transmisión vertical, con muchos años por delante de coexistencia con el virus y los fenómenos inflamatorios e inmunológicos derivados de la infección, con el fin de establecer medidas preventivas que podrían resultar en una mayor calidad de vida y una mejora en la esperanza de vida. En la era de la terapia antirretroviral de alta eficacia, la infección por el VIH se ha convertido en una enfermedad crónica, y el estudio y manejo de las comorbilidades no infecciosas se han vuelto de suma importancia. En este contexto, el estudio de la población pediátrica puede además proporcionar una oportunidad única para analizar cómo los diferentes factores influyen en la enfermedad vascular subclínica, en ausencia de factores de RCV clásicos.

En este contexto, los objetivos principales de esta Memoria son:

1) Analizar los valores de las GIM en una cohorte de niños y adolescentes infectados por el VIH, en comparación con controles sanos.

2) Investigar los posibles factores (clínicos, epidemiológicos, inmunovirológicos e inflamatorios) asociados con un aumento del GIM.

3) Analizar la funcionalidad de las lipoproteinas de alta densidad en el contexto de la infección por el VIH, y su posible papel en el desarrollo de enfermedad cardiovascular.
4) Identificar posibles marcadores de complicaciones no asociadas a SIDA en esta población.

Se diseñó un estudio multicéntrico, observacional, prospectivo, en el que se incluyeron, entre junio y diciembre de 2011, niños y adultos jóvenes en seguimiento en los hospitales que integran la Cohorte de Madrid de niños y adolescentes infectados por el VIH, así como controles sanos de características comparables. El estudio fue aprobado por el Comité de Ética e Investigación Clínica de los seis hospitales participantes. Todos los participantes y/o sus padres o tutores legales dieron su consentimiento informado por escrito para participar en el estudio.

Los criterios de exclusión incluyeron infecciones agudas u oportunistas, enfermedades inflamatorias crónicas, diabetes, enfermedad renal, hipertensión y antecedentes familiares de enfermedad cardiovascular Los voluntarios sanos fueron reclutados prospectivamente como controles entre los hermanos sanos de los pacientes infectados por el VIH, niños no infectados nacidos de madres infectadas por el VIH, voluntarios sanos de un instituto de enseñanza secundaria perteneciente a la misma zona urbana y niños que asistían al hospital para someterse a procedimientos de cirugía menor o a la consulta de pediatría general. Eran criterios de exclusión adicionales para controles sanos la presencia de enfermedades infecciosas o inflamatorias, enfermedades crónicas y el consumo de medicamentos. Los controles fueron incluidos con los objetivos de lograr un grupo con características comparables en cuanto a edad, sexo, origen étnico e índice de masa corporal (IMC) ($\pm 1 kg/m^2$).

Los datos se recogieron de forma prospectiva, mediante entrevista con la familia del paciente y el pediatra, y se complementaron con los datos registrados en la base de la Cohorte de Madrid. A todos los participantes se les realizó un examen físico completo, incluyendo medidas antropométricas y de tensión arterial mediante técnicas estandarizadas. Simultáneamente, se determinó el GIM mediante ecografía, utilizando para el análisis la media de dos determinaciones a nivel de la pared posterior de la arteria carótida común (1 cm proximal a la bifurcación). Las imágenes se digitalizaron para su análisis posterior utilizando un procedimiento automatizado. Se extrajeron muestras de sangre para proceder a determinar

perfil lípidico, insulina y glucosa, por métodos enzimáticos estándar en los respectivos hospitales participantes. Una muestra de cada participante fue enviado BioBanco-VIH, situado en el Hospital General Universitario Gregorio Marañón e integrado en la Red de Investigación en SIDA (RIS), que se encargó de su procesamiento y congelación a -80 º para determinaciones posteriores. Las determinaciones incluyeron biomarcadores inflamatorios y cardíacos; PCR, interleucina 6 , mieloperoxidasa , ligando CD40, molécula de adhesión vascular soluble (sVCAM), proteína quimio atrayente de monocitos (MCP- 1), P-selectina y CD14 soluble, y se llevaron a cabo en un único laboratorio de forma centralizada. La expresión de marcadores de activación y senescencia en células T se midieron por citometría de flujo a partir de muestras frescas o criopreservadas. En un subgrupo de pacientes, se estudió la función anti-inflaatoria de las lipoproteínas de alta densidad (HDL) ex vivo mediante ensayos de migración de monocitos (MCA).

Los resultados de nuestros estudios corroboran, en la mayor serie de pacientes analizada hasta la fecha, la hipótesis que sostiene que el GIM se encuentra incrementado en las primeras décadas de la vida durante la infección por el VIH, destacando la necesidad de un diagnóstico precoz y el tratamiento de los factores de riesgo cardiovascular en esta población única. Las técnicas ecográficas para la evaluación del GIM han demostrado ser seguras y son bien tolerados por niños y adolescentes, y pueden ser útiles para evaluar el riesgo cardiovascular en la población pediátrica. Nuestros resultados apoyan el papel de la propia infección por el VIH más que del tratamiento antirretroviral, como han sugerido otros estudios pediátricos, en el proceso aterogénico. No se detectó en nuestro estudio elevación significativa de ninguno de los biomarcadores analizados, a excepción de sVCAM, y no se encontró correlación con la GIM, lo que sugiere que su utilidad como marcadores de riesgo cardiovascular en niños y adolescentes infectados por el VIH podría ser limitada. Los fenómenos de activación y senescencia del sistema inmune están presentes desde la infancia durante la infección por el VIH, a menudo relacionados con la presencia de carga viral detectable, aunque no se encontró asociación con la presencia de aterosclerosis subclínica. En el subgrupo de pacientes incluidos en el estudio de funcionalidad de las lipoproteínas de alta densidad, la función anti-inflamatoria de las HDL estaba disminuida en los pacientes infectados por el VIH por vía perinatal, especialmente en presencia de carga viral detectable,

y en relación a una situación de mayor inflamación y mayor activación inmune. Estos resultados sugieren que, a pesar del TAR, la situación de inflamación / inmunoactivación scunadria a la infección puede comprometer la funcionalidad de HDL, contribuyendo al proceso aterosclerótico. Por lo tanto, estrategias terapéuticas orientadas a aumentar la cantidad de las HDL con actividad anti-aterogénica conservada pueden ser prometedoras a la hora de mejorar la calidad de vida de los sujetos infectados por el VIH. Por último, en un intento por identificar marcadores pronóstico accesibles a la práctica clínica, nuestro estudio analizó la relación entre el cociente CD4/CD8 y los fenómenos de inflamación y activación inmune. Nuestros resultados demuestran que un cociente CD4/CD8 bajo se asocia a frecuencias más altas de activación (HLA-DR + CD38 +), senescencia (CD28 - CD57 +) , y agotamiento inmunológico (HLA-DR + PD- 1 +) de células T, y a alteraciones en las subpoblaciones linfocitarias, con predominio de células de memoria. Estos hallazgos sugieren que la persistencia de un cociente CD4/CD8 invertido a pesar del TAR podría ser un marcador útil para identificar a los sujetos infectados por el VIH por transmisión vertical con mayores niveles de activación y senescencia del sistema inmune, y por lo tanto mayor riesgo de envejecimiento prematuro y aparición de complicaciones no asociadas a SIDA.

En resumen, los resultados recogidos en esta Memoria demuestran que los sujetos infectados por el VIH de forma perinatal presentan aterosclerosis carotidea precoz, en comparación con controles no infectados. Las técnicas ecográficas no invasivas resultan seguras y bien toleradas y permiten el estudio de enfermedad vascular subclínica en población pediátrica. Los niños y adolescentes infectados por el VIH presentan niveles más altos de activación y senescencia inmune, a menudo relacionados con la presencia de carga viral detectable. En ausencia de medidas preventivas específicas, lograr la supresión viral y un adecuado control de las alteraciones metabólicas asociadas al TAR deben ser una prioridad en el manejo de estos pacientes, con el fin de prevenir las enfermedades cardiovasculares en el futuro. La inversión del cociente CD4/CD8 podría permitir identificar a los sujetos en situación de riesgo, y por tanto éste debe ser un marcador a considerar en cuanto al diseño de estrategias orientadas al diagnóstico y tratamiento de los fenómenos de inflamación y activación inmune, en la búsqueda de una mayor calidad y esperanza de vida en la población de pacientes infectados por el VIH por transmisión vertical. .

SUMMARY

SUMMARY

The World Health Organization estimates that 34 million people were living with HIV by the end of 2012. Of them, 2.5 million were children[1]. During 2012, 350.000 children were infected by HIV and 250.000 died from AIDS related causes. Access to antiretroviral treatment has dramatically changed life expectancy of HIV-infected children, reducing the incidence of opportunistic infections, HIV-encephalopathy, morbidity and mortality. However, the data show that life expectancy of HIV-infected individuals remains lower than the one of general population[2], mainly because a well described increase in non-AIDS related diseases, a group of conditions generally associated to premature aging, including cardiovascular disease (CVD), renal impairment, hepatic disease, osteoporosis and non-AIDS defining malignancies[3].

Among other associated morbidity, HIV-infected patients show an increase in atherosclerosis at relatively young age, and as the mean age of HIV-infected individuals is progressively increasing, CVD is likely to gain further importance as a cause of mortality in years to come[4]. Despite being the focus of intense investigation, the etiology of the increased cardiovascular risk in this population remains unclear, most probably because of a multifactorial pathophysiology. A pro-inflammatory and pro-oxidative status secondary to the infection itself, as well as to bacterial translocation and immune activation, together with the undesirable effects of antiretroviral therapy and classical cardiovascular disease factors have been suggested as the main drivers of the accelerated atherogenic process during HIV infection[5]. The development of atherosclerosis begins early in life[6], and the study of subclinical atherosclerosis in children and adolescents offers the opportunity to clarify the specific role of the different suggested factors on the atherosclerotic burden in the absence of classical CVD risk factors.

Several non-invasive approaches have been suggested for detection of HIV-infected patients at higher risk for developing CVD, such as combination of multiple biomarkers or direct measurement of arterial stiffness, endothelial function or subclinical atherosclerosis (intima-media thickness –IMT). Previous studies using IMT have shown an increase of cardiovascular risk in HIV-infected children[7,8]. Some groups have also described in this population higher levels of inflammatory biomarkers, such as high sensitivity C-Reactive

protein (hsCRP), although results from different studies are controversial[7,9]. No studies to date have properly addressed the correlation between accelerated clinical aging and the presence of activated/senescent T-cells in childhood, although it has been described a higher frequency of these two phenotypes of $CD8^+$ and $CD4^+$ T-cells in HIV-infected children[10,11].

As ART-treated HIV-infected children can now expect to live for many years, identification of subjects at risk for complications related to chronic inflammation, immune activation and immunosenescence is warranted, in order to establish preventive measures potentially resulting in higher quality of life and life expectancy of this unique population.

In the era of highly effective antiretroviral therapy, HIV infection has become a chronic condition, and the study and management of non-infectious comorbidities have become of paramount importance. In this context, the study of pediatric populations may provide a unique opportunity to analyze how inflammation and immune activation influence subclinical vascular disease, in the absence of classical CVD factors.

In this context, in this Thesis we aimed to:

1) Analyze IMT values in a cohort of HIV infected children and adolescents in comparison to age-matched healthy controls
2) Investigate potential factors (clinical, epidemiological, immuno-virological and inflammatory) associated with an increase in IMT
3) Specifically address the contribution of dysfunctional high density lipoproteins to the atherogenic process and
4) Identify potential markers of non-AIDS related complications in this population.

For such a purpose, we designed a longitudinal, observational study aiming to include children and young adults integrating the Madrid Cohort of HIV-Infected Children and Adolescents, as well as healthy uninfected controls with comparable characteristics. The study was reviewed and approved by the Ethics Committee and Clinical Research of the six participating Hospitals. All participants, parents or legal guardians and children over twelve years, gave written informed consent to take part in the study.

Participants were recruited between June and December 2011. Exclusion criteria included acute or opportunistic infections, chronic inflammatory diseases, diabetes, kidney

disease, hypertension, and family history of premature CVD. Healthy volunteers were prospectively enrolled as controls from healthy siblings of the HIV infected patients, uninfected children born to HIV-infected mothers, healthy volunteers from a high school in the same urban area and children attending the laboratory for minor surgery purposes or the general pediatric clinics. Additional exclusion criteria for healthy controls included current infectious or inflammatory illnesses, chronic conditions and current use of medications. Controls were included with the goals of achieving a group with similar age, sex, ethnicity and body mass index (BMI) (±1 kg/m²).

Data were collected prospectively, from an interview with the patient's family and the managing paediatrician when necessary, together with a thorough revision of medical records. All children underwent physical examination, including anthropometric and blood pressure measurement by standardized techniques. Simultaneously, IMT was determined using ultrasonography. Two measurements were made at the common carotid artery (1 cm proximal to the bifurcation). Far wall images were obtained and digitalized for each patient by a single experienced technician provided with portable equipment. Posterior measurements were performed using commercial software by a blind cardiologist. Immuno-virological details and previous ART history were collected from the Cohort of Madrid collaborative Pediatric HIV Study database. Fasting blood samples were drawn, for real-time measurements of insulin and glucose levels as well as lipid profile, by standard enzymatic methods at the participating hospitals. A sample of every participant was sent to the Pediatric HIV BioBank integrated in the Spanish AIDS Research Network (RIS), processed and stored at -80º using standard procedures for subsequent determinations. Determinations included inflammatory and cardiac biomarkers; CRP, Interleukin 6, myeloperoxidase, CD40 ligand, soluble vascular adhesion molecule (sVCAM), monocyte chemo attractant protein (MCP-1), P-selectin and soluble CD14, and were performed in a central lab. T-cell activation and senescence were measured by immunophenotyping performed from fresh samples or cryopreserved peripheral blood mononuclear cells, thawed using methods that have been optimized and validated. In a subgroup of patients, functionality of high-density lipoproteins (HDL) was assessed *ex vivo* by monocyte chemotaxis assay.

Results of our studies corroborate in a large number of patients the hypothesis that IMT is already increased in the first decades of life during HIV infection, highlighting the need of a prompt diagnosis and treatment of cardiovascular risk factors in this unique population. Ultrasound techniques for evaluation of IMT are safe and well tolerated and can be useful to evaluate cardiovascular risk in pediatric populations. Our findings support a role for the HIV infection itself on the atherogenic process, rather than for antiretroviral treatment as suggested by other pediatric studies. No significant elevation was detected for any of the studied biomarkers, except for sVCAM, and no correlation was found with IMT, suggesting that its utility to screen cardiovascular risk in HIV-infected children and adolescents might be limited. Immune activation and senescence are present since childhood during HIV infection, often related to persistence of detectable viral loads, although no direct relation to subclinical atherosclerosis could be demonstrated. In the subgroup of patients included in a pilot study aiming to explore High-density lipoproteins anti-inflammatory properties, HDL were dysfunctional in perinatally HIV infected patients, especially in the presence of detectable viral loads, and associated to increased levels of inflammation and immunoactivation. These results suggest that, despite ART, ongoing inflammation/immunoactivation may compromise HDL functionality, contributing to the increased atherosclerotic burden described in HIV-infected patients. Thus, new therapeutic approaches aiming to raise HDL particles with preserved anti-atherogenic activity might be a promising strategy to improve the quality of life of HIV-infected subjects. Finally, we explore the CD4/CD8 ratio as a predictor of increased immunoactivation and immunosenescence in a subgroup of vertically-HIV infected children and adolescents. We found that a low CD4/CD8 ratio correlated with higher frequencies of activated (HLADR+CD38+), senescent (CD28-CD57+), and exhausted CD8+ T cells (HLADR+PD-1+), and a skewed T-cell phenotype from naïve toward effector memory. These findings suggest that persistance of an inverted CD4/CD8 ratio despite ART might be a useful marker to identify individuals with increased levels of immune activation / senescence, and thus increased risk of premature ageing and non-AIDS events, among the population of vertically infected subjects.

In summary, this Thesis highlights the fact that vertically HIV-infected subjects show higher carotid atherosclerosis compared to controls, which can be diagnosed using non-

invasive techniques. HIV-infected children and adolescents already display higher levels of T cell activation and senescence, often related to the presence of detectable viral load. Viral suppression and optimal metabolic control are mandatory in order to prevent future cardiovascular disease, while specific preventive measures are implemented. Diagnosing and targeting immune activation might be crucial in order to increase quality of life and life expectancy of the very unique population of perinatally HIV-infected children.

INTRODUCCIÓN

EPIDEMIOLOGÍA DE LA INFECCIÓN POR VIH

Según datos del último informe conjunto de la Organización de las Naciones Unidas (ONU) y de la Organización Mundial de la Salud (OMS), publicado a finales de 2012, se estima en 34 millones el número de infectados por el Virus de la Inmunodeficiencia Humana (VIH) en el mundo[1]; de ellos 2,5 millones son niños menores de 15 años. En dicho año, 350.000 jóvenes menores de 15 años se infectaron y 250.000 fallecieron como causa directa o indirecta de la infección. La situación resulta especialmente dramática en la región del África subsahariana, donde se encuentra el 90% de los infectados por el VIH.

En España, desde el inicio de la epidemia en los años ochenta hasta Junio de 2012, se han notificado un total de 82.009 casos de SIDA, de los cuales 966 han sido declarados en pacientes pediátricos[12]. En 2011 se diagnosticaron un total de 2.763 nuevos casos de infección por el VIH. La principal vía de trasmisión fueron las relaciones sexuales sin protección, principalmente entre hombres que mantenían relaciones con hombres (54%), seguida de contactos heterosexuales (31%). En la actualidad, tan sólo un 5% de los nuevos diagnósticos se debe al uso de drogas por vía parenteral. Debido a la generalización de las medidas de prevención de la transmisión vertical en nuestro medio, en 2011 sólo se diagnosticaron un total de 8 casos de infección por el VIH en niños por transmisión materno-fetal. Sin embargo, desde el año 1997 hasta la actualidad, se ha producido un aumento progresivo en el número de nuevos diagnósticos pediátricos en niños nacidos fuera de España, que actualmente representan el 30% de los pacientes pediátricos en seguimiento en nuestro país[13].

La Comunidad de Madrid notificó en el año 2011 un total de 778 casos de infección por VIH, es decir, un 28.1% de los casos totales en España. Entre ellos, se comunicaron 2 casos de infección por transmisión vertical.

INFECCIÓN POR EL VIH Y TRATAMIENTO ANTIRRETROVIRAL

El VIH se identificó como agente causante del Síndrome de la Inmunodeficiencia Humana (SIDA) en 1983. Desde entonces, se han invertido millones de dólares en investigación, para intentar comprender cómo se transmite, cómo penetra en las células, qué mecanismos desencadena y, en definitiva, cómo causa enfermedad. Probablemente sabemos más de la patogenia de esta enfermedad, actualmente, que de ninguna otra enfermedad crónica, lo que constituye sin duda un modelo de investigación translacional.

Desde la aparición del tratamiento antirretroviral (TAR), tanto la morbilidad como la mortalidad de los pacientes con infección por el VIH con acceso a tratamiento han disminuido drásticamente, convirtiendo la infección por el VIH en una enfermedad crónica[14,15]. Este hecho, inicialmente descrito en adultos, es una realidad también en población pediátrica[16–18]. La ausencia de datos sobre tolerancia y seguridad en niños de las nuevas combinaciones de antirretrovirales condicionaron en un primer momento una restricción en su uso a pacientes más graves, muchos de ellos en estado de inmunodepresión severa. Sin embargo, los espectaculares beneficios en cuanto a reducción de morbilidad y mortalidad fueron de magnitud tal que obligaron a la generalización del TAR en población pediátrica en un brevísimo plazo de tiempo. Lamentablemente, los tratamientos que tan eficazmente demostraron ser capaces de disminuir la replicación viral permitiendo la recuperación del sistema inmune y frenando el avance de la enfermedad, no han logrado hasta la fecha la erradicación del virus. Con el paso del tiempo, los efectos beneficiosos han quedado además oscurecidos en parte por el incremento de complicaciones relacionadas con el tratamiento prolongado, entre las que destaca por su importancia la toxicidad metabólica.

Paralelamente al aumento de la supervivencia y a la disminución de las infecciones oportunistas y tumores derivados del estado de inmunodepresión que acompañaban clásicamente a esta enfermedad y que marcaban el pronóstico, se ha descrito en población adulta un importante aumento de lo que se conoce como "mortalidad no asociada a SIDA"[3]. Los pacientes infectados por el VIH sufren un proceso de inflamación crónica que conduce a un envejecimiento prematuro, y padecen un grupo de enfermedades asociadas a la edad tales como el deterioro cognitivo, la enfermedad hepática, cardiovascular, renal, o tumoral, que se han convertido a día de hoy en la principal amenaza para su supervivencia.

En esta década, son tres las prioridades en el campo de la infección por el VIH; la búsqueda de un mecanismo de erradicación de la infección, la generalización del acceso al tratamiento para todos los sujetos infectados, y la profundización en el conocimiento de los mecanismos fisiopatológicos que propician el proceso de envejecimiento prematuro condicionando el pronóstico de la enfermedad[19]. Este último aspecto es aún más importante en el caso de los pacientes infectados por transmisión vertical, cuyo sistema inmune se desarrolla ya en presencia de la infección, y con muchos más años por delante durante los cuales el virus continuará ejerciendo su acción sobre el organismo. Es por tanto lógico suponer que los posibles efectos del envejecimiento prematuro tendrán mayor repercusión en esta población. El estudio de los fenómenos inmunológicos asociados a la infección crónica por el VIH en población pediátrica, además de una necesidad, supone una oportunidad única para entender los mecanismos fisiopatológicos que condicionan la enfermedad por el VIH.

INFECCIÓN POR EL VIH Y ENFERMEDAD CARDIOVASCULAR

Entre los fenómenos emergentes asociados al envejecimiento prematuro descritos en la era post-TAR, el incremento de complicaciones cardiovasculares es sin duda, por su impacto epidemiológico, uno de los más relevantes. Numerosos estudios en los últimos años han descrito la existencia de una asociación entre la infección por VIH y la enfermedad cardiovascular. Pocos años después de la introducción del TAR, y paralelamente al aumento de supervivencia que supusieron los nuevos fármacos antirretrovirales, se publicaron los primeros artículos describiendo un discreto pero preocupante aumento en estos pacientes de eventos cardiovasculares[20,21]. Debido a la generalización de los nuevos tratamientos y a la disminución en la incidencia de enfermedades oportunistas y tumorales, los pacientes vivían más, y la edad media de la población de enfermos infectados por el VIH aumentaba. Parecía lógico por tanto que apareciesen enfermedades propias del envejecimiento, como los accidentes cerebrovasculares e infartos de miocardio, en esta población que por otro lado con mayor frecuencia se encontraba expuesta a factores cásicos de riesgo cardiovascular (RCV) tales como el tabaquismo, y que anteriormente no llegaba a edad de padecerlos. Sin embargo, pronto resultó evidente que la incidencia de la enfermedad cardiovascular superaba la

esperada teniendo en cuenta incluso estos factores, según datos extrapolados de población general[21]. Paralelamente a la disminución en la mortalidad, se había producido un aumento en los tiempos de exposición a los antirretrovirales, puesto que los tratamientos debían mantenerse y la enfermedad se había cronificado, y con este aumento en la exposición aumentaron también los efectos secundarios y las complicaciones metabólicas, tales como la lipodistrofia, las alteraciones lipídicas, la resistencia a la insulina y el síndrome metabólico. En este contexto, el aumento en la mortalidad cardiovascular fue atribuido inicialmente al propio tratamiento y sus complicaciones metabólicas[21–23]. Aunque los investigadores insistían de manera general en que los beneficios superaban ampliamente los riesgos del tratamiento en estos pacientes, la asociación del tratamiento antirretroviral con un incremento de eventos cardiovasculares modificó la ecuación de riesgo-beneficio, obligando a la comunidad científica a plantearse la cuestión de cuál era el momento idóneo para iniciar tratamiento y si éste debía mantenerse de por vida. Posteriormente, y debido entre otros al estudio SMART de interrupción de tratamiento guiado por el recuento de CD4[24], que demostró que la disminución en la mortalidad asociada al TAR se debía no sólo a la disminución de la incidencia de enfermedades oportunistas y SIDA, sino también en gran parte a la prevención de enfermedades no asociadas a SIDA (y en concreto a la disminución en la incidencia de enfermedad cardiovascular), se sabe que los beneficios compensan ampliamente los riesgos del tratamiento a largo plazo.

Éste y otros estudios posteriores demostraron que si bien la exposición al TAR, y especialmente a algunas familias, puede constituir un factor de RCV, el VIH *per se* también tiene efecto sobre el sistema cardiovascular, y que ambos factores se relacionan de forma independiente con el riesgo aumentado de sufrir eventos cardiovasculares desfavorables[25–27]. Los fenómenos fisiopatológicos subyacentes han sido objeto de múltiples estudios, y sin embargo no son del todo conocidos. Muy recientemente, un gran número de trabajos sostienen que la situación de inflamación crónica[28] y los fenómenos de activación inmune crónica e inmunosenescencia secundarios a la infección[29,30], podrían ser parcialmente responsables del envejecimiento prematuro descrito en esta población, contribuyendo a la aceleración de la enfermedad vascular en estos pacientes. No resulta fácil, no obstante, discernir en qué proporción contribuye a dicho RCV cada uno de los factores implicados; el

propio VIH, el estado de activación inmune e inflamación crónica secundarios a la infección; el TAR y sus consecuencias metabólicas; y los factores clásicos de RCV, generalmente muy prevalentes en la población infectada por el VIH[31].

En este sentido, el estudio de población pediátrica nos ofrece una oportunidad única para analizar los efectos independientes de la infección y su tratamiento en el sistema cardiovascular, en ausencia de los factores de RCV clásicos. En los últimos años, diferentes estudios utilizando marcadores subrogados, han demostrado que los niños y adolescentes infectados por el VIH de forma perinatal ya presentan signos de afectación vascular subclínica[7,8,32,33]. Sin embargo, los resultados no han sido en absoluto concluyentes en cuanto al análisis de los factores potencialmente implicados, sugiriendo en algunos casos relación entre el aumento de riesgo cardiovascular y la presencia de CV detectable[9], la situación inmunológica o la exposición a diferentes familias de fármacos antirretrovirales[7,8].

FISIOPATOLOGÍA DE LA ATEROSCLEROSIS

Aunque las manifestaciones clínicas de la arteriosclerosis no se presentan hasta la edad adulta, sabemos que el proceso de aterogénesis comienza en edades tempranas de la vida[6,34]. Numerosos estudios han demostrado que muchos de los factores de riesgo cardiovascular afectan tanto al funcionamiento del endotelio vascular como a la estructura de la pared arterial desde la primera década de la vida[35–37]. Aunque no se conoce con exactitud la fisiopatología de la arteriosclerosis, actualmente se sabe que se trata de una enfermedad inflamatoria crónica, en la que mecanismos inmunes interaccionan con factores de riesgo metabólicos e inician, mantienen y activan lesiones a nivel del endotelio arterial[38]. En sentido estricto, se trata de un proceso natural; una respuesta protectora a los daños sufridos por el endotelio y las células musculares de la pared arterial, consistente en la formación de lesiones fibrosas y grasas, precedidas y acompañadas de inflamación. Las lesiones avanzadas que pueden llegar a ocluir la luz arterial no serían sino el resultado de un desequilibrio en los mecanismos de regulación del proceso inflamatorio, consecuencia de una respuesta fibroproliferativa descontrolada ante las diversas formas de daño endotelial[39].

La primera lesión reconocible del proceso arteriosclerótico es la estría grasa, formada por una agregación de macrófagos y células T ricas en lípidos que atraviesan la íntima arterial. Estas lesiones aparecen de manera ubicua en la mitad de las autopsias realizadas a niños de entre 10 y 14 años de edad[6]. Posteriormente, estas lesiones pueden evolucionar, convirtiéndose en placas fibrosas de mayor complejidad, compuestas por capas de células de músculo liso, macrófagos, y linfocitos T, muchos de los cuales se encuentran activados, como demuestra la expresión del marcador HLADR[40]. La **figura 1** muestra el proceso de formación de la placa de ateroma.

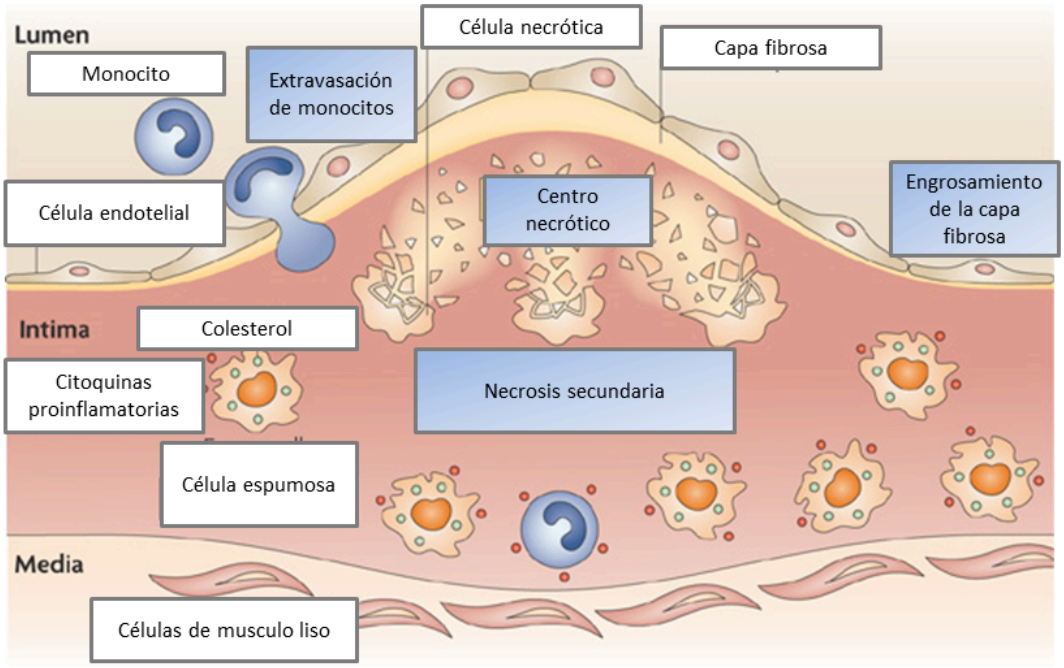

Figura 1: El proceso de formación de la placa de ateroma.
Adaptado de *Nature*. 2002 Dec 19-26; 420(6917):868-74. Inflammation in atherosclerosis. Libby P[41]

Lejos de jugar un papel pasivo, las células endoteliales desempeñan un papel principal en este proceso. En respuesta al paso de macrófagos y células T al espacio subendotelial, las células del endotelio secretan sustancias reguladoras del tono vascular y mediadores inflamatorios que contribuirán a la formación de la placa de ateroma[38,42,43]. Este engrosamiento de las capas íntima y media de la pared de las medianas y grandes arterias se

considera el primer cambio anatómico del proceso de arteriosclerosis[44]. Estas lesiones, que se organizan en torno a un centro de lípidos y tejidos necróticos recubierto por una capa de tejido conectivo, pueden crecer hasta ocluir la luz del vaso. La mayor parte de las muertes por infarto agudo de miocardio se producen sin embargo por desestabilización de la placa, por fisuras o rupturas en el margen de la capa fibrosa, que originan hemorragias masivas en el interior de la placa, trombosis y oclusión arterial[45].

Actualmente se sabe que dichos cambios en la estructura de la pared arterial no son definitivos, sino que pueden revertirse con una adecuada intervención y una mejora del estilo de vida (hábitos dietéticos y ejercicio), especialmente durante la edad pediátrica[46,47].

En el contexto de la infección por el VIH, el aumento de riesgo de enfermedad cardiovascular puede deberse a diferentes causas, como veremos a continuación. En primer lugar, se ha descrito que la infección por el VIH afecta a la pared arterial y tiene capacidad por sí misma de acelerar el proceso aterosclerótico[48,49]. En segundo lugar, la infección crónica por el VIH se acompaña de una situación de inflamación sistémica crónica, a la que cada día se da mayor importancia en la patogenia de la aterosclerosis y de los eventos cardiovasculares[38,50–53]. En tercer lugar, las alteraciones metabólicas que frecuentemente presentan estos pacientes, secundarias al tratamiento, pueden conducir a la aparición de dislipemia, disfunción endotelial, hipercoagulabilidad, activación plaquetaria y disminución de la fibrinólisis[22,23].

EFECTO DEL VIH PER SE EN EL PROCESO ATEROSCLERÓTICO

Estudios realizados en muestras procedentes de autopsias demuestran que en el paciente infectado por el VIH, la arteriosclerosis presenta unas características histológicas únicas, similares a las descritas en el contexto del rechazo crónico en trasplantados[54]. Las lesiones progresan de forma rápida, con una gran proliferación de células musculares lisas, originando protrusiones endoluminales. Todo ello sugiere que la fisiopatología del desarrollo de la lesión ateromatosa difiere en presencia del VIH. Algunos de los mecanismos fisiopatológicos propuestos se resumen a continuación.

En primer lugar, los linfocitos infectados tienen mayor capacidad de penetración en el espacio subendotelial que los no infectados[54,55]. La explicación a este fenómeno podría ser un aumento de permeabilidad del endotelio vascular inducido por la proteína viral *Tat*[56]. El enriquecimiento del medio de cultivo de células endoteliales con proteína *Tat* ha demostrado además aumentar la expresión de citoquinas, y en particular de la proteína quimioatrayente de monocitos (*monocyte chemoattractant protein-1*-MCP-1) y de moléculas de adhesión, como la molécula de adhesión vascular VCAM-1, incrementando el paso de macrófagos y monocitos al espacio subendotelial[57]. Estos monocitos infectados por el VIH que penetran en el espacio subendotelial expresan un fenotipo particular, con gran cantidad de receptores CCR2 en su superficie, que además de ser receptor para MCP-1, es correceptor del VIH. Estudios realizados en tejidos procedentes de autopsias de sujetos infectados han demostrado que el VIH en la pared arterial no sólo se localiza en el espacio subendotelial, sino que también afecta a las células musculares lisas arteriales, que también expresan en su pared celular CCR-2[58]. Por otro lado, las proteínas virales *Tat* y *gp120* inducen en el endotelio vascular un aumento en la producción de endotelina-1, el vasoconstrictor más potente conocido[59].

De manera adicional, la proteína viral *Nef* es capaz de inhibir el receptor ABCA-1, al que se unen las lipoproteínas de alta densidad (HDL), mediadoras del transporte reverso de colesterol[60]. Así, las HDL no son capaces de extraer colesterol de las células, contribuyendo a su acumulación y por tanto a la formación de la placa de ateroma.

EFECTO DE LA INFLAMACIÓN CRÓNICA

Como se ha descrito anteriormente, el VIH tiene capacidad de activación de diversas vías inflamatorias, muchas de las cuales afectan directamente a la pared vascular[61]. Como consecuencia de la infección, se produce un aumento en la liberación de citoquinas y la expresión de moléculas de adhesión endotelial que facilitan la adherencia y transmigración de los leucocitos. Se han encontrado concentraciones plasmáticas superiores del Factor de Necrosis Tumoral Alfa (TNF-α) y de Interleuquina 6 (IL-6) en pacientes con infección por el VIH respecto a sujetos seronegativos, y se ha descrito una asociación entre ambas citoquinas y la carga viral del VIH, tanto en adultos[62] como en niños[9]. Los niveles de la molécula de

adhesión intercelular ICAM-1, la molécula de adhesión vascular VCAM-1 y el factor von Willebrand también se han encontrado significativamente aumentadas en pacientes con infección VIH[9,33]. En adultos nunca tratados (naïve), respecto a sujetos seronegativos, se ha observado además una disminución de sus concentraciones plasmáticas paralela al descenso de carga viral al iniciar tratamiento, tanto con pautas con Inhibidores de la Proteasa (IP) como con Inhibidores de la Transcriptasa Inversa No Análogos de Nucleósidos (ITINAN)[63]. En el estudio SMART, las concentraciones basales elevadas de IL-6, dímeros-D y Proteína C reactiva (PCR) se asociaron con un riesgo aumentado de mortalidad por todas las causas. En los pacientes que interrumpían el tratamiento hubo un aumento en las concentraciones de IL-6 y de dímero-D, y este incremento en los biomarcadores en el grupo de tratamiento intermitente se correlacionaba positivamente con la CV del VIH[24]. Éste y otros estudios son la base sobre la que se sustenta la hipótesis de que la inflamación juega un papel determinante en la enfermedad cardiovascular durante la infección por el VIH, que a su vez es, al menos parcialmente, responsable de gran parte de los fenómenos asociados al envejecimiento en esta población. La **Figura 2** esquematiza el papel de la inflamación en el daño vascular en la infección por el VIH.

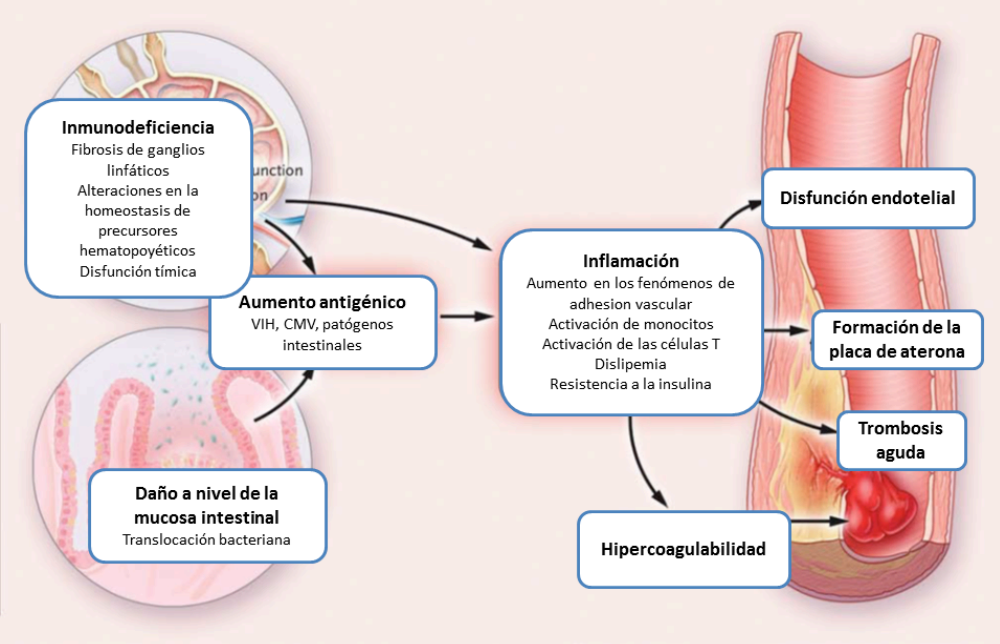

Figura 2. Papel de la inflamación en el proceso de remodelamiento vascular asociado a la enfermedad por el VIH**.** Adaptado de *NEJM*, 2013, Deeks S[64].

Numerosos investigadores han estudiado el papel de las diferentes citoquinas y mediadores inflamatorios en la enfermedad cardiovascular en el contexto de la infección por el VIH. Un gran número de publicaciones, entre ellas todas las específicamente pediátricas, utilizan la medición del índice o grosor íntima-media carotideo (GIM) como marcador de enfermedad vascular subclínica (o aterosclerosis subclínica) y por ende, de RCV, encontrando asociación con múltiples marcadores inflamatorios[65–67]. Otros grupos, en estudios epidemiológicos realizados en adultos, han demostrado directamente asociación entre marcadores inflamatorios y mortalidad de causa cardiovascular[68,69]. A continuación se describen brevemente las principales moléculas implicadas en el proceso arteriosclerótico en el contexto de la infección por el VIH.

Proteína C reactiva

La PCR se ha propuesto como un marcador que representa en cierto modo una integración funcional de la dinámica entre las diversas citoquinas que están directamente implicadas en la aterogénesis[51]. Es una proteína de origen hepático, cuya síntesis es estimulada por citoquinas como la IL-6 y el TNF-α. Tiene una vida media larga en plasma y actualmente es considerada tanto un mediador como un marcador de enfermedad aterosclerótica. Numerosos estudios epidemiológicos en población general demuestran que su elevación es un factor predictor independiente de eventos cardiovasculares futuros, independientemente de la edad, el tabaquismo, los niveles de colesterol, la presión arterial y la diabetes mellitus[51,70]. No obstante, todavía no se ha establecido un punto de corte definitorio de normalidad para esta PCR ultrasensible.

Con la creciente preocupación por el incremento de enfermedad cardiovascular en los llamados países desarrollados, se han multiplicado los intentos por encontrar marcadores que ayudasen a identificar de forma fiable a los pacientes en situación de riesgo. Múltiples grupos han analizado el papel de la PCR como predictor de enfermedad cardiovascular, y hoy por hoy, se trata de uno de los marcadores más validados de RCV. Son numerosas las publicaciones que han relacionado la elevación de la PCR con la enfermedad vascular subclínica, tanto en población general[71] como en población infectada por el VIH, en la que la PCR también ha demostrado ser un factor predictor independiente de RCV[66,68]. Sin embargo,

los estudios realizados en población pediátrica infectada por el VIH, no han arrojado resultados concluyentes. Aunque los estudios iniciales sugerían la existencia de un incremento de PCR desde edades tempranas en los pacientes infectados por el VIH[7,33], otros más recientes no han confirmado estos resultados[9,72].

Interleukina-6

La IL-6 es una citoquina que se sintetiza fundamentalmente en los linfocitos T y en los macrófagos. Es crucial para la activación leucocitaria y endotelial y también induce la síntesis de la PCR en el hígado[73]. Se expresa en ciertas zonas de la placa de ateroma, y contribuye a un aumento de la inestabilidad de la misma, al inducir a su vez metaloproteinasas que degeneran la matriz de la placa de ateroma. También induce la síntesis de MCP-1 y TNF-α[74]. En adultos, se han llevado a cabo estudios que sugieren que podría tratarse de un marcador de RCV equivalente a la PCR[75,76].

Proteína quimioatrayente de monocitos

La MCP-1 activa la migración de monocitos en respuesta a una señal inflamatoria, promoviendo entre otras cosas el paso de macrófagos hacia el espacio subendotelial. Concentraciones elevadas de MCP-1 se han asociado a una mayor mortalidad a los 10 meses de un infarto de miocardio[77], y algunos de sus polimorfismos se asocian a riesgo especialmente elevado de disfunción endotelial[78]. En niños, pocos estudios han estudiado la MCP-1 en relación a la infección por el VIH, y solo uno ha encontrado elevación de los alores del MCP-1 asociada a la infección[9].

Moléculas de adhesión

Las moléculas de adhesión, ICAM-1, sVCAM-1 y P-selectina, promueven la adhesión de los leucocitos al endotelio vascular, lo que permite la penetración de los monocitos en el espacio subendotelial. Se ha descrito un aumento de las concentraciones de estas moléculas en situaciones de inflamación crónica, y su aumento se ha relacionado con disfunción endotelial y con la presencia de factores cardiovasculares clásicos en población general[79]. En el contexto de la infección por el VIH, concentraciones elevadas de algunas de

estas moléculas se han asociado a enfermedad vascular subclínica, tanto en niños como en adultos[9,66,72].

Mieloperoxidasa

La mieloperoxidasa es una enzima producida por los leucocitos, que se libera cuando éstos se activan. Se asocia a disfunción endotelial y enfermedad coronaria en población general[80]. En población infectada por el VIH, valores altos se han asociado a enfermedad vascular subclínica en algunos estudios en adultos así como en estudios pediátricos[8,66].

Se han estudiado muchas otras moléculas en relación la génesis, progresión o desestabilización de la placa de ateroma, como el factor von Willebrand o el Fibrinógeno, sin embargo, hasta la fecha no existe consenso en cuanto a su posible utilidad como predictores de enfermedad cardiovascular.

La mayor parte de los estudios mencionados se refieren a población adulta. En población pediátrica, pocos y muy recientes trabajos han analizado el papel de los marcadores anteriormente descritos en la disfunción vascular en el contexto de la infección por el VIH. Entre ellos, cabe destacar los estudios publicados por McComsey et al. y Miller et al. Además de encontrarse entre los primeros investigadores en constatar la existencia de un aumento de GIM en niños y adolescentes infectados por el VIH, el grupo de McComsey ha descrito también su asociación con algunos de los marcadores de inflamación y moléculas de adhesión anteriormente descritas[8,33]. Más recientemente, las publicaciones de Miller et al. han ampliado el conocimiento acerca de los biomarcadores asociados a la infección por el VIH de trasmisión vertical, aunque sus trabajos no incluyen ningún marcador subrogado de arteriosclerosis subclínica y/o función vascular. Resulta imprescindible profundizar en el estudio concreto del proceso aterogénico en el contexto de la infección vertical por el VIH, con unas características inmunológicas que la diferencian de la infección de adquisición no vertical[81], y que además, en el caso concreto de la enfermedad vascular, nos ofrece la posibilidad de analizar el proceso en ausencia de los factores clásicos de RCV.

A día de hoy, en el manejo de la infección por el VIH, el enfoque clínico continúa basándose en la optimización de dos marcadores subrogados de progresión de la enfermedad: el recuento de linfocitos T CD4 y la carga viral. Sin embargo, en los pacientes con acceso a

TAR, la preocupación por conseguir y mantener cargas virales indetectables, y reducir la incidencia de enfermedades oportunistas van poco a poco cediendo su lugar en la práctica clínica al manejo de otras complicaciones asociadas a la infección, y la necesidad de monitorización de la inflamación asociada a la enfermedad es cada día más real. Algunos de los estudios mencionados sugieren que algunos regímenes de antirretrovirales pueden tener un mayor efecto sobre determinados mediadores inflamatorios elevados en la infección por el VIH[69,82], hallazgo que tendría importantes implicaciones terapéuticas. Estos datos refuerzan el cuerpo de evidencia que sostiene que en un futuro próximo el manejo de la enfermedad por el VIH requerirá la monitorización de nuevos marcadores que reflejen el nivel de inflamación y activación del sistema inmune, que podrían convertirse en próximas dianas terapéuticas en el intento de controlar el envejecimiento prematuro asociado a la infección.

EFECTO DEL TRATAMIENTO ANTIRRETROVIRAL

Ya se ha comentado anteriormente cómo tras la introducción de la terapia antirretroviral combinada se describió un aumento de incidencia de eventos cardiovasculares desfavorables. Inicialmente este hecho se asoció con las complicaciones metabólicas del tratamiento, y especialmente con la exposición prolongada a fármacos de la familia de los Inhibidores de Proteasa (IP), con conocidos efectos sobre el metabolismo lipídico y de la glucosa. Estudios posteriores que han tenido en cuenta la presencia de alteraciones metabólicas, tabaquismo y otros factores de riesgo, han confirmado que los fármacos antirretrovirales pueden constituir factores independientes de RCV [83,84]. El papel del TAR en la generación, progresión o desestabilización de la placa de ateroma, puede deberse a su vez a diferentes mecanismos. En primer lugar, algunos autores han descrito el efecto pro-oxidativo de la terapia combinada[85,86], especialmente cuando incluye IP[87], y se sabe que el estrés oxidativo es uno de los factores promotores y aceleradores del proceso arteriosclerótico en población general. También algunos análogos de los nucleósidos, como el abacavir o la didanosina, se han asociado a un incremento de RCV, al parecer por su efecto pro inflamatorio[88]. El abacavir, además, se ha relacionado directamente con disfunción endotelial[89]. Por otro lado, estudios in vitro han demostrado que los IP aumentan la expresión

del *scavenger receptor* o receptor CD36*,* que expresado en la pared de los macrófagos del espacio subendotelial, se encarga de captar lipoproteínas modificadas[90]. Así, el macrófago comienza a fagocitar lipoproteínas ávidamente, acumulando ésteres de colesterol en su interior y transformándose en una célula espumosa o *foam cell.* Como hemos visto anteriormente, la apoptosis de estas células y la coalescencia de sus depósitos de colesterol dará lugar a la formación del núcleo lipídico de la placa de ateroma.

La formación de las células espumosas está limitada por la cantidad de lipoproteínas modificadas presentes en el entorno. Cuanto más tiempo permanezcan las lipoproteínas de baja densidad (LDL) en circulación, más posibilidades tendrán de ser modificadas. Los IP inhiben la expresión del receptor de LDL y, por tanto, afectan al mecanismo de eliminación de estas lipoproteínas[91]. El transporte reverso de colesterol mediado por las HDL debería contrarrestar esta situación extrayendo colesterol de las células espumosas, pero su receptor, como se ha mencionado anteriormente, se ve inhibido por la proteína viral *Nef*. Ambos factores condicionan que en la infección por el VIH, el transporte reverso de colesterol se vea seriamente afectado[92].

EFECTO DE LA ACTIVACIÓN Y SENESCENCIA DEL SISTEMA INMUNE

Los mecanismos que incrementan el riesgo de enfermedad cardiovascular en los pacientes con infección por el VIH, con y sin tratamiento, son como se ha descrito complejos, no del todo conocidos, y probablemente se encuentran tremendamente interrelacionados. Por un lado se mezclan factores de RCV clásicos, tales como el sedentarismo o el tabaquismo, con efectos producidos directamente por el propio VIH o derivados de la toxicidad de los antirretrovirales. La enfermedad arteriosclerótica es un proceso inflamatorio, en el que desde el inicio de la formación de la placa tanto células endoteliales como macrófagos y células T juegan un papel fundamental. Por otro lado, se sabe que la infección crónica por el VIH desencadena un estado de inflamación y activación del sistema inmune crónicos, que solo son revertidos parcialmente por la terapia antirretroviral[93]. La activación de las células T, y especialmente de los linfocitos T CD8 se ha asociado de forma independiente al riesgo de progresión de la enfermedad en los pacientes no tratados[94,95] y también a la recuperación inmunológica y evolución clínica en los pacientes que inician terapia[96]. La existencia de fundada evidencia acerca de la relación entre

inflamación, activación inmune y escasa ganancia de linfocitos T CD4 que se produce en los pacientes en tratamiento, pese a lograr supresión viral[96], contribuyó al nacimiento de la hipótesis que sostiene que la activación del sistema inmune secundaria a la infección juega un papel fundamental en el desarrollo de las patologías asociadas al envejecimiento prematuro en estos pacientes, y entre otras, especialmente en la enfermedad cardiovascular **(Figura 3)**.

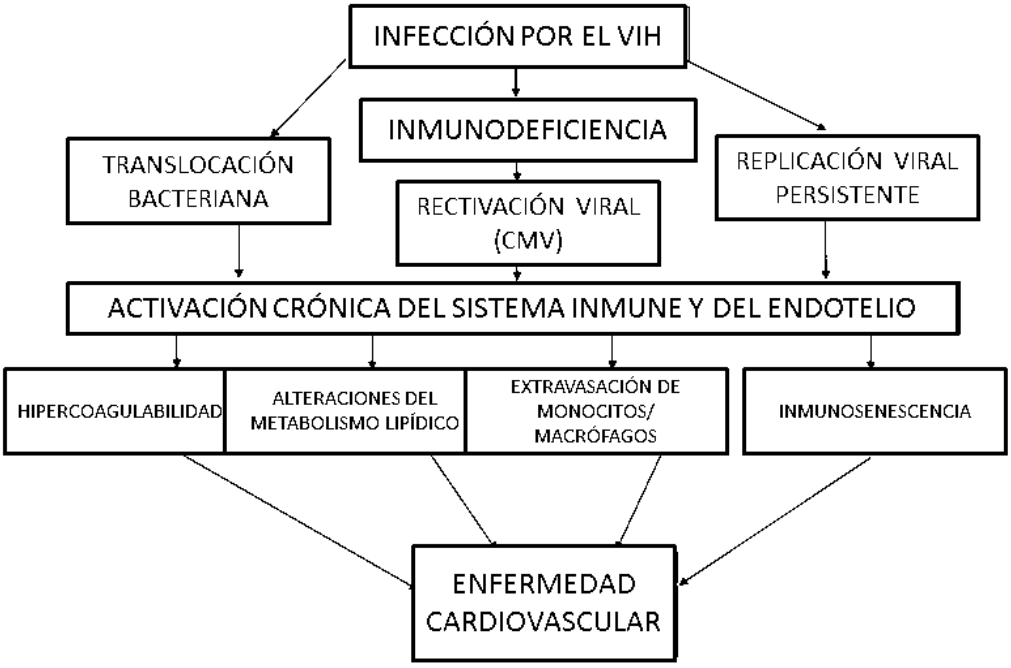

Figura 3: Bases inmunológicas de la Enfermedad Cardiovascular asociada a la Infección por el VIH. Adaptado de P. Hsue, *The Journal of Infectious Diseases,* 2012[97].

El papel que desempeñan las células T activadas y sus diferentes productos inflamatorios en el desarrollo de la arteriosclerosis ha sido bien estudiada y descrita en población general como se ha mencionado anteriormente[98]. Sin embargo, pocos trabajos han analizado esta relación en el contexto de la infección por VIH. En este sentido, los trabajos más significativos corresponden al grupo de la Universidad de San Francisco, California, y demuestran que recuentos bajos de linfocitos T CD4 constituyen un factor independiente de

RCV en individuos infectados por el VIH[99]. Más recientemente han sugerido que tanto la activación como la senescencia de las células T secundarias a la infección por el VIH se asocian con la presencia de placas de ateroma[30]. Dicha asociación, más evidente en pacientes con CV detectable, se mantiene en los pacientes tratados que alcanzan la supresión virológica, apoyando la tesis anteriormente expuesta que sostiene que el TAR logra tan solo una recuperación funcional parcial del sistema inmune. Otros trabajos de este mismo grupo sugieren que en los pacientes infectados por el VIH, la coinfección por citomegalovirus (CMV) y la presencia de células T específicas frente a CMV se relacionan con mayor GIM[100,101]. De ello se deduce que la respuesta inmune a CMV media de alguna manera el proceso aterosclerótico. La explicación a dicho fenómeno podría venir de la mano del también recientemente fenómeno descrito del envejecimiento prematuro del sistema inmune o inmunosenescencia. Hay numerosos trabajos que sugieren que las infecciones víricas, y entre ellas la infección por el VIH y por CMV, podrían actuar como aceleradores del proceso de envejecimiento del sistema inmune[29,102–104]. El mecanismo subyacente sería un agotamiento prematuro del sistema inmune, como resultado de la presencia viral en el organismo. A ello se suma en el caso del VIH la disfunción tímica asociada a la enfermedad, que condiciona una disregulación en la homeostasis de las células T, y una disminución de células T reguladoras, que son las responsables de frenar la activación. Estas células activadas comenzarían un proceso de expansión clonal en respuesta a los antígenos persistentes, y como resultado se generarían y acumularían abundantes células muy bien diferenciadas, disfuncionales, pero capaces de liberar moléculas inflamatorias que contribuirían a mantener la situación de inflamación crónica y así al desarrollo de las enfermedades no asociadas al SIDA[29] (**Figura 4**).

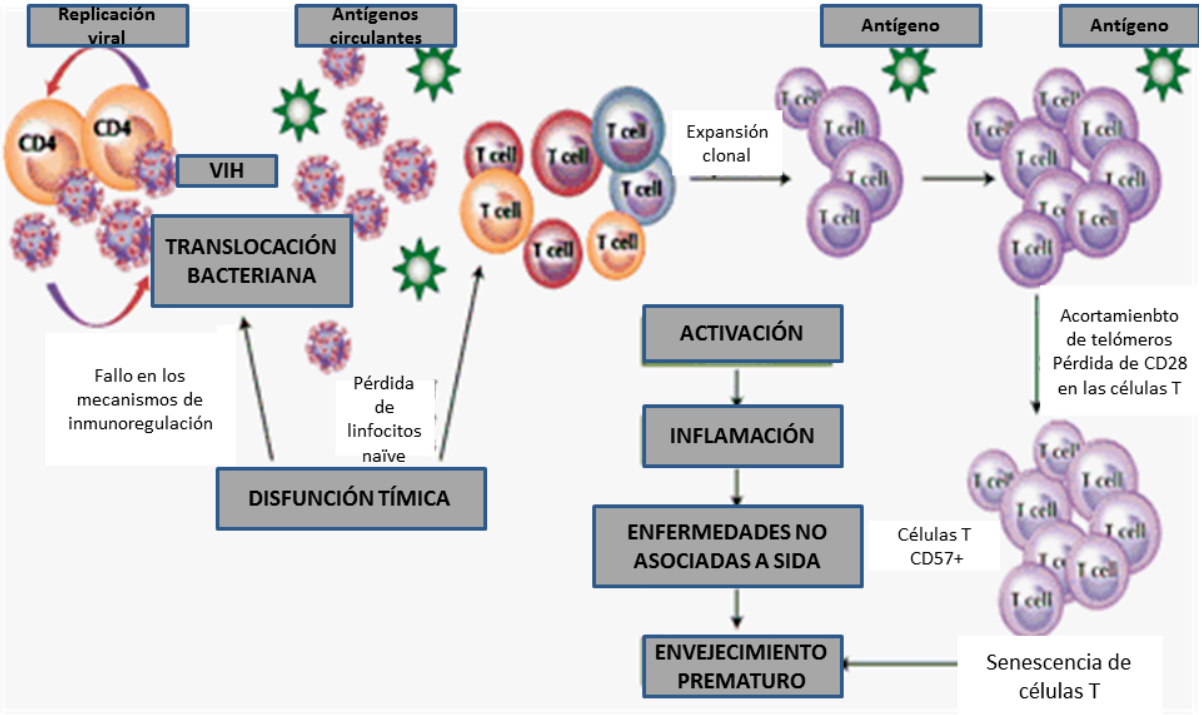

Figura 4: Senescencia en la infección por el VIH, adaptado de *Current HIV/AIDS reports*, 2010, Desai S at al [29].

En el caso concreto de la infección por el VIH, múltiples los factores podrían contribuir a los fenómenos de activación crónica y senescencia del sistema inmune. A los efectos de la replicación viral residual, le persistencia de reservorio viral y las coinfecciones, habría que sumar el recientemente descrito mecanismo de la translocación bacteriana[105], que se comenta a continuación.

Algunos grupos han abordado el estudio de marcadores inflamatorios y enfermedad cardiovascular incipiente en población pediátrica infectada por el VIH, pero muy pocos han estudiado los fenómenos de activación y senescencia inmunológica en este colectivo. Que se sepa, ninguno hasta la fecha ha analizado su posible papel en el proceso de formación de la placa de ateroma. Se desconoce si las alteraciones en la homeostasis de las

células T, defectos en su diferenciación y anomalías en su funcionalidad asociadas a la infección por el VIH se producen en mayor o menor medida en un sistema inmune inmaduro y que se desarrolla en presencia del virus, como ocurre en los pacientes infectados por transmisión vertical. Trabajos previos, incluidos los de nuestro grupo, apuntan a que, efectivamente, el fenómeno de la inmunosenescencia ya se presenta en edades tempranas de la vida en los pacientes infectados por el VIH por transmisión vertical[10,11], aunque existe controversia acerca de las implicaciones clínicas que estas alteraciones a nivel de la células T pueden tener en población pediátrica[106,107].

EFECTO DE LA TRANSLOCACIÓN BACTERIANA Y LA ACTIVACIÓN DE LA INMUNIDAD INNATA

Algunas de las más recientes publicaciones analizando las bases inmunológicas de la enfermedad cardiovascular durante la infección por el VIH, sugieren que, entre otros factores como la coinfección por CMV y los fenómenos de activación y senescencia del sistema inmune, los mecanismos de inmunidad innata podrían contribuir de forma importante al proceso de formación y progresión de la placa de ateroma. Los monocitos, precursores de los macrófagos presentes en las placas ateromatosas, se encuentran entre los principales liberadores de citoquinas inflamatorias conocidos[108]. Algunos autores sugieren que su papel en cuanto a la progresión de la enfermedad por el VIH y aparición de eventos no asociados al SIDA puede ser comparable, si no superior, a la de las células T[109,110]. Dado el protagonismo de estas células en cuanto al desarrollo de las lesiones vaculares, numerosos estudios en los ultimos años han analizado su papel en el contexto de la infección por el VIH. Se sabe que polimorfismos en el gen que codifica la MCP-1 se asocian a mayor grosor y mayor progresión del GIM en los pacientes con el VIH[78] y que la activación monocitaria, medida por la determinación de los valores plasmáticos de CD163, que se ha descrito asociada a la infección por el VIH tanto en pacientes en tratamiento como sin tratar, se relaciona con la presencia de placas coronarias no calcificadas[111]. Otros marcadores de activación monocitaria, como el CD14 soluble (sCD14), ya se habían asociado anteriormente a progresión de la enfermedad y mortalidad en los pacientes infectados[112], en relación al fenómeno de la traslocación bacteriana

Durante la primoinfección por el VIH, se produce una intensa replicación viral a nivel del tejido linfoide primario de la mucosa intestinal (MALT), con una enorme pérdida de linfocitos T CD4 a ese nivel, apoptosis de células epiteliales y pérdida en la integridad de la mucosa intestinal[113,114]. Como consecuencia de estas alteraciones en la barrera mucosa intestinal, se produce un paso de productos bacterianos al torrente sanguíneo, que al igual que los antígenos virales circulantes, actúan como estimulantes de la activación del sistema inmune. Los valores de lipopolisacárido bacteriano (LPS), uno de los productos más frecuentemente utilizado como marcador de translocación bacteriana desde el intestino, están aumentados en los pacientes infectados por el VIH, y que sus valores aumentan tras la interrupción del tratamiento antirretroviral, sugiriendo que éste tiene un efecto al menos parcial en la recuperación del MALT[114–116]. Además, se ha descrito una elevación de los niveles de LPS asociada a activación monocitaria en determinadas complicaciones secundarias a la infección, como la demencia asociada al VIH[117], y se ha visto que algunos marcadores de activación de monocitos como el sCD14, que aumentan en respuesta a la presencia de LPS en sangre, están elevados también en la infección por el VIH, y se asocian directamente con la aparición de fenómenos no SIDA y mortalidad[112].

Actualmente, la translocación bacteriana se considera uno de los mecanismos fundamentales responsables de los procesos de inflamación y la activación inmune asociados a la infección por el VIH, y por tanto, parcialmente responsables de las complicaciones no asociadas a SIDA en estos pacientes. Numerosos investigadores han destacado la importancia de desarrollar estrategias orientadas al control de la translocación bacteriana, asi como también a la disminución de la inflamación y la activación inmune[118,119]. Por el momento no se ha establecido su relación con la enfermedad vascular, aunque algunos estudios han demostrado una disminución en precursores endoteliales paralela al aumento en LPS en voluntarios sanos[120], y asociación entre estos marcadores de activación monocitaria y factores de coagulación, así como alteraciones del metabolismo lipídico y de la insulina, y por ende enfermedad cardiovascular[121,122]. Curiosamente, muy pocos grupos de investigación han estudiado estos fenómenos en el contexto de la infección vertical por el VIH. Puesto que la infección de transmisión vertical posee características inmunológicas muy diferentes a las de la infección de transmisión horizontal[81], los conocimientos adquiridos en población adulta no son necesariamente transferibles a los niños. Un estudio reciente ha descrito la presencia

de valores altos de LPS en pacientes de transmisión vertical en los primeros seis meses de vida[123,124]. Sin embargo, los resultados del único estudio que ha analizado su posible relación con la enfermedad cardiovascular no han resultado concluyentes[125].

ALTERACIONES DEL METABOLISMO DE LÍPIDOS

El TAR se asocia en ocasiones a alteraciones metabólicas importantes, y en concreto a cambios en el metabolismo de los lípidos, que pueden incrementar de manera importante el riesgo de enfermedad cardiovascular. Las dislipemias más frecuentemente asociadas al tratamiento antirretroviral en niños son la hipertrigliceridemia, el aumento en las cifras de colesterol total y el descenso en los niveles de lipoproteinas de alta densidad[126,127]. Aunque la contribución de la hipertrigliceridemia al desarrollo de enfermedad cardiovascular parece bastante relativa, no hay duda acerca del papel del colesterol y las lipoproteinas de alta y, especialmente, baja densidad[128]. Las HDL son partículas con conocidas propiedades antiaterogénicas, lo que implica participación en múltiples actividades biológicas, como el transporte reverso del colesterol, acción directa sobre las células endoteliales, acción antioxidante, antitrombótica y antiinflamatoria. Muy recientemente, se ha descrito que las lipoproteínas ApoA-I y ApoA-II, que forman parte estructural de las HDL, poseen además funciones antimicrobianas y antivirales, por lo que la impresión actual es que las HDL constituyen una familia heterogénea de partículas que sirven de plataforma para que se ensamblen proteínas que cumplen un papel importante en el metabolismo de lípidos y también en la inmunidad innata.

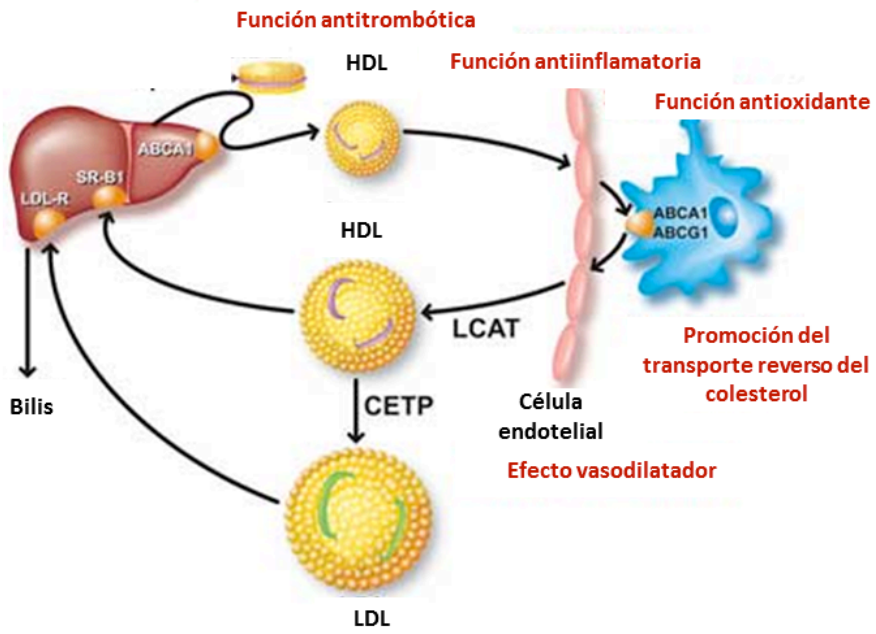

Figura 5. Función de las Proteínas de Alta Densidad (HDL). Adaptado de Clinical Laboratory News, 2007. "HDL-C; The Changing Testing Paradigm". Alan T. Remaley[129].

Sin embargo, en determinadas circunstancias, y en concreto ante situaciones de inflamación y en presencia de algunas infecciones, las HDL pueden sufrir un cambio transformacional y perder estas características, convirtiéndose en partículas pro-inflamatorias[130]. La pérdida de las funciones antiinflamatorias de las HDL podría jugar un papel importante en el caso de la enfermedad por VIH, como se ha visto que sucede en otras infecciones[131,132] y, sin embargo, muy pocos estudios han analizado el papel de las HDL en el contexto de la infección por el VIH, y ninguno que se sepa en población pediátrica. En 2008, Rose et al describieron una disminución de la funcionalidad de las HDL en los pacientes adultos infectados por VIH en cuanto a menor capacidad de transporte reverso de colesterol[133] , pero sus propiedades anti-inflamatorias no fueron evaluadas.

VALORACIÓN DEL RIESGO CARDIOVASCULAR MEDIANTE MÉTODOS NO INVASIVOS

El desarrollo de la placa de ateroma es un proceso lento, que comienza en la primera o segunda década de la vida[6]. El aumento creciente en la incidencia y prevalencia de enfermedad cardiovascular en los países del llamado primer mundo ha generado un interés creciente por la búsqueda de herramientas que permitan una detección precoz del proceso de remodelamiento arterial, o lo que es lo mismo, una detección de la enfermedad vascular en fase subclínica. La identificación precoz de los sujetos en situación de RCV posibilitaría la selección de los sujetos susceptibles de beneficiarse de un programa de prevención de eventos cardiovasculares, mejorando así la calidad de vida y la supervivencia a medio y largo plazo.

Los procedimientos ecográficos disponibles en la actualidad permiten evaluar la estructura de la pared arterial, así como la rigidez o funcionalidad del endotelio vascular, de manera no invasiva. La medición del grosor de las capas íntima y media arteriales a nivel de la carótida (GIM o *intima-media thickness – IMT)* es sin duda el marcador subrogado de RCV que se ha estudiado más extensamente. Empezando por la descripción de la técnica por Pignoli et al. en 1986[134], lo que empezó siendo una técnica experimental se ha convertido en el principal, más accesible y más validado marcador de RCV[135,136]. El GIM es el marcador utilizado en la mayor parte de las publicaciones científicas en el campo de la imagen y la arteriosclerosis. En un metaanálisis reciente, Lorenz et al. revisaron los datos de más de 37000 sujetos, concluyendo que incrementos de 0.1 mm en el GIM suponen un aumento del riesgo de infarto de miocardio del 10-15%, y del riesgo de ictus del 13-15%[137]. Una vez demostrada su asociación con otros factores de RCV, y su capacidad de predicción del riesgo de forma independiente, la medición del GIM se recomienda hoy en día como elemento diagnóstico para su uso en la práctica clínica habitual, y de hecho, constituye el único método no invasivo recomendado por la Asociación Americana de Cardiología (*American Heart Association* - AHA) para la estimación del riesgo de desarrollo de enfermedad cardiovascular en adultos[138].

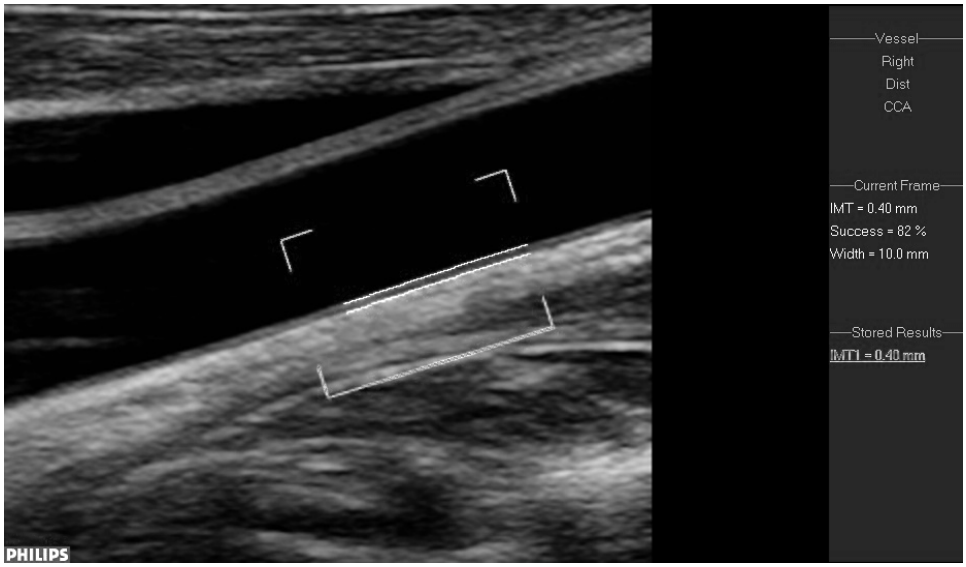

Figura 6. Medición del grosor íntima medio carotideo (GIM) mediante procedimientos ecográficos no invasivos. Imagen correspondiente a uno de los participantes del Estudio CaroVIH[139].

La ausencia de unos criterios establecidos para la medición del GIM ha dado lugar a una gran divergencia en los resultados. Este hecho queda patente en el mencionado metaanálisis realizado por Lorenz et al. en el que llama la atención la heterogeneidad metodológica[137]. Por este motivo, Touboul et al. establecieron en 2004 los "Criterios de Mannheim", en un intento por unificar los criterios para distinguir entre la presencia de una placa ateromatosa y el aumento del GIM, y para estandarizar la metodología de la medición[140,141]. Los criterios de Mannheim se resumen en el Anexo III de esta Memoria.

En el contexto de la población infectada por el VIH, existe una creciente preocupación a nivel de la comunidad científica en torno a los fenómenos no asociados a SIDA, y muy especialmente la enfermedad cardiovascular, ya que parecen ser responsables en la actualidad de la menor esperanza de vida que presenta esta población pese al tratamiento antirretroviral y el control de las infecciones oportunistas. Los modelos de predicción de RCV tales como el SCORE o Framingham, no han sido diseñados para población infectada por el VIH, ni tampoco validados en esta población, y por tanto resultaba prioritario disponer para este colectivo de herramientas más fiables de medición del RCV.

Son numerosos los estudios realizados en adultos que demuestran que el GIM está aumentado en los pacientes infectados por el VIH[100,142–145], y que han estudiado su asociación con los diferentes factores de RCV, así como con variables relacionadas con la infección y su tratamiento[31,146–149].

En el contexto de la población pediátrica, tanto las revisiones realizadas en 2006 y 2011 por Groner et al. y Lamotte et al. [46,150] respectivamente, como las guías redactadas en 2009 por Urbina et al. sobre evaluación de la enfermedad vascular subclínica mediante procedimientos no invasivos para la Academia Americana de Cardiología[151], concluyen que existen en la actualidad suficientes datos como para considerar la medición del GIM un procedimiento válido para la investigación en pediatría. Los autores inciden en la imperiosa necesidad de profundizar en el conocimiento de los valores de referencia en pediatría previo a su estandarización a la práctica clínica, variaciones según género, peso, raza, así como estudios longitudinales que analicen los cambios en función de la edad o el estadio puberal. Desde su publicación, se ha profundizado notablemente en las líneas de investigación propuestas. Uno de los estudios más relevantes es el realizado por Juonala et al. que tras revisar datos de cuatro cohortes prospectivas con un total de 4830 individuos y una media de seguimiento de 22,4 años, concluye que existe correlación entre los factores de RCV en la infancia y la elevación del GIM a partir de los nueve años de edad[152]. Estos datos responden a una de las cuestiones más inquietantes que se interponen al uso universal del GIM como herramienta diagnóstica en pediatría, y que es la dificultad para demostrar su validez como predictor de eventos cardiovasculares en edad adulta. Puesto que se trata de pacientes pediátricos, no podrá demostrarse asociación entre la medición del GIM en la infancia y la aparición de eventos cardiovasculares desfavorables hasta pasado aproximadamente medio siglo.

Son numerosas también las publicaciones que han apuntado a la disminución en el GIM tras la intervención sobre los diferentes factores de RCV en diferentes poblaciones pediátricas [47,153,154]. Estos datos, sin duda contribuyen a apoyar la validez del GIM como marcador de RCV, puesto que sugieren que el grosor de la pared arterial disminuye en paralelo a la disminución en el número de factores de RCV.

Aunque en menor proporción, se han publicado ya los primeros estudios realizados en población pediátrica infectada por el VIH, que en general apuntan a la existencia de una

elevación del GIM en los niños y adolescentes infectados por el VIH[7,8,32,33,155]. Aunque las diferencias en cuanto a GIM en los pacientes infectados descritas por los distintos grupos de investigación son bastante variables, y algunos resultados incluso contradictorios, probablemente esto se deba a la variabilidad de las técnicas ecográficas utilizadas, a la ausencia de interpretaciones estandarizadas del GIM, al reducido tamaño muestral de la mayoría de los trabajos y a dificultades en el emparejamiento de los pacientes con controles sanos. En general, los autores concluyen que es necesaria la realización estudios de mayor tamaño muestral, así como el seguimiento longitudinal de los pacientes, para lograr un mejor conocimiento del proceso de formación de la placa de ateroma durante la infección por el VIH de transmisión vertical.

Además de los métodos que evalúan los cambios anatómicos, como el GIM, otros métodos no invasivos que evalúan parámetros funcionales de rigidez arterial se han utilizado para estudiar la enfermedad vascular subclínica. Algunos de los más conocidos son la medición de la dilatación mediada por flujo (*Flow-mediated dilation o* FMD) o el análisis de los cambios mecánicos, distensibilidad arterial y propagación de la onda del pulso (*Arterial stiffness* - AS and *Pulse Wave Velocity* - PWV). Estos parámetros se han utilizado en estudios de investigación tanto en adultos como en niños, infectados y no infectados. Sin embargo, aún no existe consenso acerca de su utilización en pediatría, la metodología resulta compleja y el procedimiento más largo y peor tolerado por los más pequeños, y a diferencia del GIM no se encuentran entre los métodos recomendados para la valoración de RCV.

Comprender los mecanismos fisiopatológicos subyacentes al aumento en la incidencia de enfermedad cardiovascular en los pacientes infectados por el VIH es el primer paso para poder plantear una optimización de los tratamientos, perfeccionar el manejo de la enfermedad y poder ofrecer a los pacientes una calidad de vida y unas expectativas de supervivencia similares a las de la población general. Los pacientes infectados por vía vertical, cuyo sistema inmune ha convivido con el virus durante la etapa madurativa, y que en cualquier caso pronto serán los que acumulen más años de infección crónica y exposición a antirretrovirales, probablemente sean los que más se beneficien de nuevas estrategias de prevención y tratamiento de las complicaciones asociadas a la infección. Establecer

marcadores válidos de RCV y desarrollar herramientas que permitan identificar a los sujetos que padecen mayor probabilidad de sufrir eventos cardiovasculares desfavorables se presenta como uno de los grandes desafíos clínicos de la infección por VIH. En una era en la que, gracias al TAR, la infección por el VIH ha pasado a considerarse una enfermedad crónica, ha cobrado presencia en la mortalidad de estos pacientes el infarto agudo de miocardio. Actuando desde las consultas de pediatría se tiene la oportunidad de identificar a los pacientes de riesgo desde las fases más tempranas de la enfermedad, teniendo así la seguridad de llegar a tiempo de revertir el proceso mediante el desarrollo de programas de intervención precoz, que incidan en la modificación del estilo de vida, adquisición de hábitos dietéticos y de ejercicio físico saludables, e inicio del tratamiento médico en los casos que lo así lo requieran, con el propósito de reducir los eventos cardiovasculares desfavorables en esta población en un futuro próximo.

COHORTE NACIONAL DE PACIENTES PEDIÁTRICOS INFECTADOS POR EL VIH

Desde que en 1997 se instauraron los programas de prevención de la transmisión vertical, que contemplan la administración de TAR a las mujeres durante el embarazo, así como la realización de profilaxis durante el parto y en el recién nacido tras el nacimiento, los nuevos diagnósticos por contagio perinatal se han situado por debajo del 2% en nuestro país, con la consiguiente disminución en el número de infecciones pediátricas. Aunque los nuevos flujos migratorios han traído consigo nuevos diagnósticos pediátricos y la epidemiología de la infección pediátrica está cambiando, en la actualidad la mayor parte de los niños en seguimiento en España resultaron infectados durante el pico de incidencia de transmisión vertical que se registró en nuestro país en los años 80 y 90, a consecuencia fundamentalmente del uso de drogas por vía parenteral. Se trata por tanto de una cohorte compuesta a día de hoy mayoritariamente por adolescentes que han sobrevivido a la era previa al TAR, y que presentan por tanto unas características clínicas e inmunovirológicas únicas. Su seguimiento se ha centralizado en torno a la Red Nacional de Investigación en SIDA (RIS). La Cohorte Pediátrica de la Red de Investigación en SIDA (CoRISpe) es una cohorte nacional, multicéntrica, de carácter prospectivo, de seguimiento de niños y

adolescentes infectados por VIH[156]. Su objetivo es conocer la situación epidemiológica del VIH pediátrico en España, y diseñar estudios estratégicos para mejorar la situación clínica, inmunológica y virológica de los pacientes y aumentar su calidad de vida. En la actualidad, 74 hospitales de todo el territorio nacional participan en CoRISpe, que incluye 838 niños y adolescentes. Un 64,8% (536 pacientes) son menores de edad y se encuentran en seguimiento en unidades pediátricas. Un 35,2% ya ha alcanzado la mayoría de edad y ha sido transferido a unidades de adultos.

En la Comunidad de Madrid, 287 niños y adolescentes se encontraban en seguimiento en el año 2012, constituyendo la Cohorte de Madrid de seguimiento de niños y adolescentes infectados por el VIH, integrada dentro de CoRISpe. Se trata de una cohorte iniciada en 2003, prospectiva, y en la que participan 9 hospitales públicos de la Comunidad. En esta cohorte se incluyen todos los nuevos diagnósticos en edad pediátrica, y se recogen los datos clínicos, epidemiológicos, inmunológicos y virológicos de los niños, entre otros datos. La edad media de los pacientes en seguimiento en la actualidad es de 13,5 años, con un rango que va de los 3 meses a los 22 años. A 31 de Diciembre de 2012, 149 de estos pacientes continuaban en seguimiento en las consultas de VIH pediátrico. En la Cohorte de Madrid, y en línea con lo descrito anteriormente, la mayor parte de los nuevos diagnósticos son niños de procedencia extranjera[13]. Más del 90% de los niños se encuentran en tratamiento en la actualidad. Debido a su origen, se trata en general de pacientes pretratados, que en muchas ocasiones han recibido más de un régimen antirretroviral, pero que en su mayoría tienen buena situación clínica e inmunológica, aunque en torno a una tercera parte no mantiene cargas virales plasmáticas indetectables. La Cohorte de Madrid dispone de más 3.000 muestras biológicas secuenciales de los niños infectados por el VIH en la Comunidad de Madrid almacenadas en el BioBanco-VIH pediátrico integrado en la Red de investigación en SIDA (RIS), ubicado en el Hospital General Universitario Gregorio Marañón[157].

Es en esta población y en contexto descrito es donde se realiza el estudio de investigación cuyos resultados fundamentales se resumen en esta Memoria. Esta Memoria se compone de un total de cuatro capítulos correspondientes a cuatro publicaciones, dos de las cuales han sido ya publicadas, la tercera aceptada para su publicación y la cuarta aún se encuentra en fase revisión.

OBJETIVOS

OBJETIVOS

El objetivo general de esta Memoria es:

Estudiar la presencia de enfermedad vascular subclínica en la Cohorte de niños y adolescentes infectados por el VIH de la Comunidad de Madrid, y analizar los posibles factores clínicos, epidemiológicos e inmunovirológicos asociados.

Los objetivos específicos son:

1. Analizar la presencia de arteriosclerosis subclínica mediante procedimientos ecográficos no invasivos (medición del Índice íntima-media carotideo) en la Cohorte de seguimiento de niños y adolescentes infectados por el VIH de la Comunidad de Madrid, en comparación con un grupo de controles no infectados por el VIH.
2. Estudiar la asociación entre la presencia de enfermedad vascular subclínica y factores clínicos y epidemiológicos.
3. Investigar la existencia de los fenómenos de activación y senescencia del sistema inmune en los niños y adolescentes infectados por el VIH y estudiar específicamente su asociación con la enfermedad vascular subclínica.
4. Identificar biomarcadores inflamatorios, cardiovasculares y de translocación bacteriana útiles en la evaluación de arteriosclerosis subclínica.
5. Analizar la funcionalidad de las lipoproteinas de alta densidad en el contexto de la infección de transmisión vertical por el VIH, y su posible papel en el desarrollo de enfermedad cardiovascular.

PUBLICACIONES

CAPÍTULO 1

Arteriosclerosis subclínica, activación inmmune y otros factores implicados.

JUSTIFICACIÓN Y OBJETIVOS

En una era en la que, debido al TAR, la infección por VIH ha pasado a considerarse una enfermedad crónica, ha cobrado presencia en la mortalidad de estos pacientes el infarto agudo de miocardio. Se ha descrito en adultos infectados por el VIH un riesgo aumentado de enfermedad cardiovascular, probablemente debido a una situación inflamatoria crónica derivada de la propia infección y una situación de activación mantenida del sistema inmune, sumado a los efectos secundarios del TAR junto a factores de RCV clásicos. La arteriosclerosis es un proceso que comienza en las primeras décadas de la vida y por ello la evaluación de niños y adolescentes resulta fundamental a la hora de identificar a los sujetos en situación de riesgo. Además, el estudio de población pediátrica permite analizar el papel de los diferentes factores que intervienen en el proceso de formación de la placa de ateroma, en ausencia de factores clásicos de RCV, contribuyendo así a comprender la fisiotatología del proceso aretriosclerótico durante la infección por el VIH.

Los objetivos de este capítulo son:

- Estudiar diferencias en cuanto a grosor íntima-media a nivel de la carótida entre los niños y adolescentes infectados por VIH en la Comunidad de Madrid y un un grupo de niños de características comparables no infectado por el VIH.
- Caracterizar posibles variables epidemiológicas, clínicas e inmunovirológicas asociadas a la enfermedad vascular subclínica, y analizar específicamente el papel del propio VIH y del tratamiento antirretroviral.
- Determinar la presencia de una situación de inflamación crónica en estos pacientes, y estudiar su influencia en el proceso aterosclerótico.
- Analizar los fenómenos de activación y senescencia de células T en esta población en comparación con individuos no infectados, y estudiar su asociación con la enfermedad vascular subclínica .

CONCLUSIONES

- Entre Junio y Diciembre de 2011, 150 niños y adolescentes infectados por el VIH, entre 3 y 23 años de edad, se incluyeron en el estudio, junto con 150 controles no infectados de características semejantes. La edad media de los participantes fue de 14,8 ± 4,6 años, un 64% eran mujeres, y la mayoría de origen caucásico (74%). El 96,7% de los pacientes había adquirido la infección por transmisión vertical, y todos excepto dos se encontraban en TAR. Un 76,2% presentaba CV indetectable (<50 copias/mL) y buena situación inmunológica, con una mediana de CD4 de 777 cel/mm³ [RIQ: 557- 1079].
- Ambos grupos eran comparables en cuanto a edad, sexo, Indice de masa corporal (IMC), hábito tabáquico o frecuencia de hipertensión arterial. Sin embargo, los pacientes infectados por el VIH tenían mayor índice cintura-cadera, mayores cifras de insulina y glucemia y peor perfil lipídico, con elevación de triglicéridos y cociente colesterol total / HDL y valores de HDL disminuidos.
- Los pacientes infectados por el VIH presentaban una elevación del GIM estadísticamente significativa (0,434mm ± 0,025 vs 0,424mm ± 0,018, p<0,001). La diferencia se mantuvo al analizar exclusivamente el grupo de pacientes en supresión viral frente al grupo control.
- En el análisis multivariante, tras ajustar por posibles variables de confusión, incluyendo edad, sexo, IMC y hábito tabáquico, la infección por el VIH se asoció de forma independiente al incremento de GIM (odds ratio (OR): 2,28; intervalo de confianza (IC) 95%, 1,25-4,13; p=0,007).
- Entre las variables clínicas e inmunovirológicas asociadas a la infección por el VIH, un bajo nadir de CD4 se asoció de manera significativa a elevación del GIM (OR: 0,82, IC95%: 0.69-0,98, p=0,033). La exposición a TAR no se asociaba de forma significativa a aumento del GIM, y de hecho, la exposición a inhibidores de la proteasa rozaba la significación estadística para ser considerado factor de protección (OR: 0,89, IC95%: 0.79-1.01, p=0,072).

- No se encontró elevación estadísticamente significativa en los valores de PCR en los niños y adolescentes infectados por el VIH.
- Los pacientes infectados por el VIH presentaron valores aumentados de linfocitos T CD4 y CD8 activados (HLADR+CD38+) (p=0,002 and p=0,087, respectivamente). Sin embargo, dicha elevación no se asoció de forma significativa con un aumento del grosor ínitma-medio carotideo.
- Los pacientes con CV detectable (>50 copias/mL), presentaban además mayor frecuencia de linfocitos T CD8 senescentes (CD28-CD57+). Este aumento en el número de linfocitos con fenotipo senescente tampoco se asoció a incremento del GIM.

Subclinical Atherosclerosis and Markers of Immune Activation in HIV-Infected Children and Adolescents: The CaroVIH Study

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Background: HIV-infected adults display increased cardiovascular disease, probably driven by inflammation and immune activation. These relationships have not been addressed in vertically HIV-infected children and adolescents, a population at very high risk for long-term non-AIDS complications.

Methods: Carotid intima media thickness (IMT) was measured in a cohort of HIV-infected children and adolescents and healthy controls. C-reactive protein and markers of immune activation ($CD38^+HLA\text{-}DR^+$) and immune senescence ($CD28^-CD57^+$) were determined.

Results: One hundred fifty HIV-infected patients and 150 controls were included, 64.8% female. IMT was thicker in HIV-infected patients (0.434 mm ± 0.025 vs. 0.424 mm ± 0.018, $P < 0.001$). After adjustment by age, sex, body mass index, and smoking status, HIV infection was independently associated with thicker IMT (odds ratio, 2.28; 95% confidence interval: 1.25 to 4.13; $P = 0.007$). Among HIV-related variables, a low CD4 nadir was related to an increased IMT. Although HIV-infected subjects presented higher frequencies of activated $CD4^+$ and $CD8^+$ T cells ($P = 0.002$ and $P = 0.087$, respectively), no relation was found between IMT and inflammation, immune activation, or senescence.

Conclusions: Structural changes of the vasculature present early in vertically HIV-infected subjects as well as immune activation and senescence. These patients should be carefully monitored for the prompt detection and early treatment of cardiovascular disease.

Key Words: HIV, adolescents, cardiovascular risk factors, IMT, immune activation

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INTRODUCTION

Because patients treated with antiretroviral drugs live longer and have to deal with the complications of aging, much attention is turning to the so called "non-AIDS"–related pathologies; a group of conditions generally associated to aging, including cardiovascular disease (CVD), renal impairment, hepatic disease, osteoporosis, and non-AIDS–defining malignancies.[1] As the mean age of HIV-infected individuals is progressively increasing, CVD is likely to gain further importance as a cause of mortality in years to come.[2] Despite being the focus of intense investigation, the etiology of the increased cardiovascular risk in this population remains unclear, most probably because of a multifactorial pathophysiology.[1] Indeed, it is difficult to fully isolate the weight of the different proatherogenic factors in the presence of classical

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CVD risk factors, which are frequently overrepresented in HIV subjects with respect to the general population. In fact, classical CVD risk factors might be overshadowing the influence of other risk factors driving atherosclerosis within HIV infection.[3,4] Although this problem is of extraordinary complexity, many pathways have already been described; among them, there is mounting evidence to support that inflammation and immune activation secondary to the infection are likely to be major drivers of atherosclerosis in HIV-infected patients.[5,6] In this context, the study of subclinical atherosclerosis in children and adolescents offers the opportunity to clarify the specific role of antiretroviral treatment (ART), HIV infection, inflammation, T-cell activation, and senescence on the atherogenic process in the absence of classical CVD risk factors. Previous studies have focused on this population using the intima media thickness (IMT) as a surrogate marker of CVD risk and inflammatory biomarkers such as high-sensitivity C-reactive protein (CRP) in rather small cohorts with controversial findings.[7–9] Besides, it has been described that HIV-infected children present higher frequencies of activated and senescent $CD8^+$ and $CD4^+$ T-cell subsets,[10,11] although its association to disease progression during childhood has been questioned.[12] No studies to date have addressed the association between subclinical atherosclerosis and immune activation in this setting. To provide new insight into the relation between subclinical atherosis and HIV-associated variables, chronic inflammation, and immune activation, we performed a cross-sectional analysis in a large cohort of vertically HIV-infected children and adolescents and healthy controls.

METHODS

Study Design and Eligibility Criteria

We performed a cross-sectional analysis from an ongoing prospective, longitudinal, multicenter observational study evaluating cardiovascular risk in a cohort of HIV-infected children and healthy uninfected controls. Study participants are children and adolescents attending the clinics of 6 different hospitals integrated in the Madrid Cohort of Pediatric HIV-infected children and adolescents. Participants were recruited between June and December 2011. Exclusion criteria included acute or opportunistic infections, chronic inflammatory diseases, diabetes, kidney disease, hypertension, and family history of premature CVD. Healthy volunteers were prospectively enrolled as controls from healthy siblings of the HIV-infected patients, uninfected children born to HIV-infected mothers, healthy volunteers from a high school in the same urban area, and children attending the laboratory for minor surgery purposes or the general pediatric clinics. Additional exclusion criteria for healthy controls included current infectious or inflammatory illnesses, chronic conditions, and current use of medications. Controls were included with the goals of achieving a group with similar age, sex, ethnicity, and body mass index (BMI) (± 1 kg/m^2).

The study was reviewed and approved by the ethics committee and clinical research of the 6 participating hospitals. All participants, parents, or legal guardians and children older than 12 years gave written informed consent to take part in the study.

Clinical Assessments

Data were collected prospectively, from an interview with the patient's family and the managing pediatrician when necessary, together with a thorough revision of medical records. All children underwent physical examination, including anthropometric and blood pressure measurement, after recommendations of the American Heart Association.[13] Weight, height, BMI, and hypertension were adjusted using z score according to the age and gender.[14,15] Immunovirological details and previous ART history were collected from the Cohort of Madrid collaborative Pediatric HIV Study database. Time with detectable viral load (VL) summarizes total time (years) with detectable VL during patient's life span. To study the effect of treatment, years of antiretroviral exposure have been analyzed as a continuous variable.

Laboratory Assays

Fasting blood samples were drawn from the 150 patients and 97 controls for real-time measurements of insulin and glucose levels and lipid profile [total cholesterol, high-density lipoprotein cholesterol (HDLc), low-density lipoprotein cholesterol] that were determined in the different participating hospitals using standard enzymatic methods. Insulin resistance was calculated using the homeostasis model assessment of insulin resistance (HOMA-IR = fasting insulin (microU/mL) × fasting glucose (g/dL)/405).[16]

In the HIV-infected group, plasma HIV-1 VL was quantified using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Inc, Branchburg, NJ) with a detection limit of 50 copies per cubic millimeter.

Absolute and percentage of CD4 and CD8 T-cell counts were concomitantly measured with standard flow cytometric methods. A sample of every participant was sent to the Pediatric HIV BioBank integrated in the Spanish AIDS Research Network (RIS) processed and stored at $-80°$ using standard procedures for subsequent determinations.[17]

T-cell activation and senescence were measured by immunophenotyping performed at the Immune-Biology Laboratory of the Gregorio Marañón Hospital, from fresh samples or cryopreserved peripheral blood mononuclear cells, thawed using methods that have been optimized and validated. Peripheral blood mononuclear cells were stained using different monoclonal antibodies: CD45-RO-phycoerythrin-Cy7 (PE-Cy7) (Becton Dikinson, San Jose, CA), CD3-allophycocyanin-Cy7 (APC-Cy7), CD4-peridinin chlorophyll protein complex, CD8-PE-Cy7, CD38-PE, HLA-DR-APC, CD57-fluorescein isothiocyanate, and PD-1-PE (Beckman Coulter, Miami, FL). Cellular activation was characterized by $HLA\text{-}DR^+$ and $CD38^+$ expression and senescence by $CD28^-$ $CD57^+$ expression. Stained cells were run on a Gallios flow cytometer (Beckman Coulter, Inc, Münster, Germany) and data analyzed using Kaluza software (Beckman Coulter, Inc).

High-sensitivity CRP was analyzed from frozen samples with a commercial instant ELISA kit (eBioscience, Inc,

San Diego, CA), with a detection threshold of 3 pg/μL, and an intraassay and interassay coefficient of variation of 6.9% and 13.1%, respectively.

Carotid Artery Ultrasound

IMT was examined using ultrasonography (CX50 portable equipment, Philips Medical Systems, Inc, Eindhoven, the Netherlands). Specific IMT detection software was previously calibrated using QLab (Philips Medical Systems, Inc, Eindhoven, the Netherlands).[18] Measurements were made bilaterally at the common carotid artery (1–2 cm proximal to the bulb) following the Mannheim criteria.[19] More than 400 measurements of a 10-mm segment of the far wall of the artery were performed and digitalized for each patient, and the median value was used for the statistical analysis as previously described.[20] Images were read by an experienced cardiologist blind to the HIV status. Median value of the right and left measurement was then calculated and used for the analysis. All carotid ultrasounds were performed by 2 trained technicians who had previously participated in a pilot study (repeated and blinded measurements performed in a random sample of 36 uninfected healthy children and adolescents) that showed no evidence of systematic observer bias. The intraclass correlation coefficient was >0.90.

Data Analyses

Qualitative variables were reported as a frequency distribution, whereas normally distributed quantitative variables were described as mean and SD. The continuous nonnormally distributed variables were reported as median and interquartile ranges (IQRs). Means for variables with

TABLE 1. Main Characteristics of Both Cohorts

	HIV-Infected Subjects (N = 150)	Control Subjects (N = 150)	*P*
Demographic data			
Age (yrs)*	14.9 (2.5–23.8)	14.7 (2.9–22.6)	0.635
Female (%)	64	64	—
Whites (%)	73.3	84	0.072
Cardiovascular risk factors			
Systolic blood pressure (mm Hg)	108 (11.2)	110 (15)	0.148
Diastolic blood pressure (mm Hg)	62 (8.4)	63.7 (10.5)	0.370
Hypertension (%)	2.7	6.9	0.107
Smokers (%)	17	12	0.256
BMI (kg/m^2)	19.8 (3.8)	20.4 (3.6)	0.276
z score BMI†	0.00 (−1 to 0.5)	0.00 (−0.5 to 1)	0.018
Waist (cm)	69.9 (10.8)	68 (9.9)	0.490
Hip (cm)	80 (13)	85 (13.9)	<0.001
Waist-to-hip ratio	0.88 (0.7)	0.81 (0.9)	<0.001
Laboratory measurements			
Glycemia (mg/dL)	87 (9.4)	82 (10.4)	<0.001
Homeostasis model assessment of insulin resistance index†	34.9 (21.6–61.7)	20.4 (10.5–38.2)	0.001
Total cholesterol (mg/dL)	174 (38.2)	164.93 (34.1)	0.053
HDLc (mg/dL)	49.8 (12.4)	60.5 (13.9)	<0.001
LDLc (mg/dL)	100.1 (30.9)	88.8 (33)	0.009
Total cholesterol/HDLc	3.7 (1.1)	2.9 (0.9)	<0.001
Triglycerides (mg/dL)†	107 (103–150)	69.5 (54–91.2)	<0.001
CRP (mg/L)†	0.10 (0.10–0.33)	0.15 (0.01–0.61)	0.197
IMT (mm)	0.434 (0.025)	0.424 (0.018)	0.001
HIV-related parameters			
On ART (%)	96.7		
Vertical transmission (%)	96.7		
HVC coinfection (%)	3.33		
VL <50 copies/mL (%)	76.4		
CD4 count (cell/mL)†	777 (557–1079)		
Nadir CD4 (cell/mL)†	359 (190–561.5)		
Cumulative exposure to PI (y)†	8.2 (3.2–11.3)		
Cumulative exposure to NNRTI (yrs)†	2.9 (0.0–7.0)		
Cumulative exposure to NRTI (yrs)†	12.6 (7.2–14.8)		
Time with detectable VL (yrs)†	10.79 (7.1–14.7)		

*Expressed as mean (range).
†Expressed as median (IQR).
Results are expressed as mean (SD), except otherwise specified. Hypertension: systolic blood pressure >p95 of reference values.
LDLc, low-density lipoprotein cholesterol; NNRT, nonnucleoside analog reverse transcriptase inhibitors; NRTI, nucleoside analogue reverse transcriptase inhibitors.

a normal distribution were compared using the Student *t* test. Nonparametric variables were examined using the Mann–Whitney and Kruskal–Wallis tests. Given the small dispersion of the independent variable—IMT—and the absence of pediatric reference values; we classified subjects as having increased IMT when they showed a value above the healthy subjects' median (0.42 mm). Simultaneous independent associations between IMT and a subset of independent variables, including cardiovascular risk factors (sex, age, tobacco use, and BMI) and HIV seropositivity were evaluated by logistic regression analysis. Then, a second multivariate analysis was performed only in HIV-infected patients to explore the independent associations with IMT. This logistic regression model included the following variables: lipodystrophy, CD4 nadir, time with detectable VL, and cumulative exposure to ART and to protease inhibitors (PI), CD4 and CD8 T-cell count. To corroborate results, the multivariate analysis was reapplied to nonsmoker perinatally infected children. Finally, linear and logistic regression models were built to explore associations between IMT, high-sensitivity C-reactive protein (hsCRP), and the percentage of HLA-DR$^+$ CD38$^+$ and CD28$^-$ CD57$^+$ T cells.

All statistical analyses were performed using the SPSS 18.0 statistical package (SPSS, Inc, Chicago, IL). The level of significance for all analyses was set at 0.05.

RESULTS

Study Population and Between Group Comparison of Clinical Characteristics

The study population included 150 HIV-infected children and adolescents and 150 healthy volunteers. Main characteristics of both cohorts are summarized in Table 1. Globally, mean age was 14.8 ± 4.6 and range was 2.5–23.8. Most subjects were female (64%) and of white origin (78%). Most of HIV-infected patients (96.7%) had acquired HIV from mother-to-child transmission and all except 4 were receiving ART at the time of study evaluation. However, only 76.2% had achieved viral suppression. Median VL for the unsuppressed group was 4607 copies/mL (230–27,600) and median logVL 3.66 copies/mL (2.36–4.44). HIV infection characteristics are described in Table 1.

HIV-infected and -uninfected subjects showed similar age, gender, blood pressure, and frequency of hypertension according to z score adjusted by age and height. Although both groups had similar BMI, z score adjusted BMI was higher in the control group. On the contrary, waist-to-hip ratio was higher in the HIV-infected group. In addition, HIV-infected patients displayed significantly higher glycemia and homeostasis model assessment scores and a worse lipid profile, with decreased levels of HDLc and higher low-density lipoprotein cholesterol, total cholesterol/HDLc ratio, and triglycerides. None of the subjects had been previously diagnosed with diabetes, hypertension, or family history of premature CVD, and none was taking hypoglycemic agents, antihypertensives, statins, or fibrates. No patient was taking trimethoprim–sulfamethoxazole.

Between Group Comparison of IMT Measurements

First of all, we compared IMT between HIV-infected and -uninfected subjects. IMT was higher in the HIV-infected children compared with the HIV-uninfected group ($P < 0.001$; Fig. 1A) in both unadjusted and adjusted analysis. This difference remained statistically significant when the analysis included only those patients on stable ART who had achieved undetectable VL ($P < 0.001$; Fig. 1B). To specifically address the effect of viral suppression, we compared IMT between virally suppressed and unsuppressed patients, and no statistically significant differences were found ($P = 0.349$; Fig. 1B). A multivariate analysis was built to simultaneously analyze the effect of diverse exposure variables on IMT. After adjustment by age, gender, tobacco use, BMI, non-HDL cholesterol, and triglycerides, HIV status was the only variable independently associated with higher IMT ($P = 0.007$; Table 2), demonstrating its independent predictive value, with a borderline significance for BMI ($P = 0.057$).

Subsequently, a second multivariate regression logistic analysis was performed, including only HIV-infected patients and adjusting by specific HIV-related variables. A lower CD4 nadir was the only variable associated to a higher IMT (Table 3; odds ratio (OR): 0.82 per 100 cell/mL increase; $P = 0.033$), with a borderline statistical significance for PI exposure (Table 3; OR: 0.89; $P = 0.072$). To corroborate these results, a subanalysis restricted to nonsmokers and perinatally infected children was done. Again, a lower CD4 nadir was associated to higher IMT (Table 3; OR: 0.77; $P = 0.019$) and PI exposure reached statistical significance (Table 3; OR: 0.84; $P = 0.023$). Results of both analyses are shown in Table 3.

Between Group Comparison of High-Sensitivity CRP Measurements

CRP was determined in a representative subgroup of 64 HIV-infected patients (51 aviremic and 15 viremic) and 30 controls. No differences were found when analyzing HIV-infected subject versus HIV-uninfected controls (Table 1) or patients with detectable versus undetectable VL. No subject in the study showed markedly increased levels of CRP, suggesting acute infection. Finally, no statistical association was found between IMT measurement and CRP values (data not shown).

TABLE 2. Multivariate Analysis: Independent Associations With Increased IMT (>p50, 0.42 mm)

Variable	OR	95% CI	*P*
HIV+	2.28	1.25–4.13	0.007
Male	1.30	0.75–2.28	0.357
Age (yrs)	0.97	0.91–1.05	0.484
Smoker	0.97	0.40–2.30	0.930
BMI (Kg/m^2)	1.09	1.00	0.057
Triglycerides	1.00	1.00	0.930
Non-HDLc	1.00	1.00	0.556

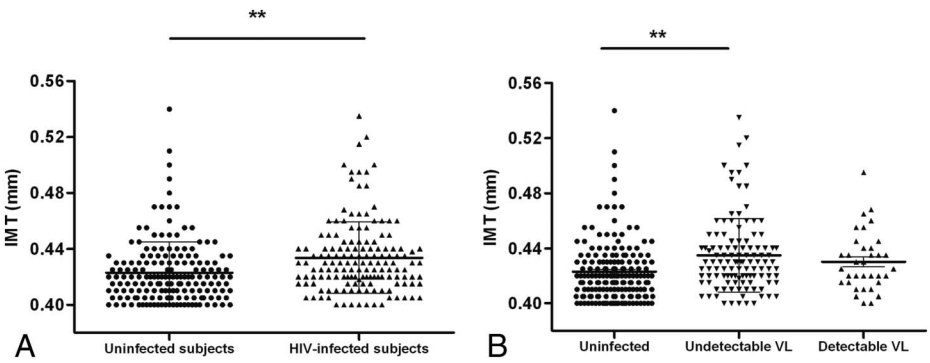

FIGURE 1. IMT (mean and SD) in HIV-infected patients and HIV-uninfected controls. A, IMT was higher in the HIV-infected group ($P < 0.001$). B, This difference remained statistically significant when the analysis included only those patients on stable ART who had achieved undetectable VL ($P < 0.001$). No differences were found among the 35 patients with detectable VL and the 115 patients that had achieved undetectable VL ($P = 0.349$). *P* values were computed using the Student *t* test.

Between Group Comparison of T-Cell Activation and Senescence Markers

To analyze influence of immune activation on the atherogenic process, T-cell activation and senescence markers were studied in a random subgroup of 11 controls and 38 patients (29 aviremic and 9 viremic) that underwent immunophenotyping by flow cytometry. This sample was representative of the subjects of the study (median age: 16.7 years, 66.7% women, 70.1% white, all of them vertically HIV-infected and on treatment).

Compared with uninfected subjects, HIV-infected patients had higher levels of HLA-DR$^+$ CD38$^+$ CD4 T cells (Fig. 2A). This difference remained significant when the comparison included only those HIV-infected patients on ART who had achieved undetectable VL (Fig. 2B). Differences in the frequency of activated CD8 T cells did not reach statistical significance (Fig. 2C). However, viremic patients showed significantly higher frequency of HLA-DR$^+$ CD38$^+$ CD8 T cells (Fig. 2D). The frequency of CD28$^-$ CD57$^+$ T cells was similar between HIV-uninfected and HIV-infected subjects, both for CD4 and CD8 subsets (Figs. 2E, G). Again, viremic patients showed an increase in the percentage of CD28$^-$ CD57$^+$ CD8 T cells (Fig. 2H).

We did not detect any significant association between markers of T-cell activation/senescence and IMT in the univariate analysis. Also, linear and logistic regression models were built to explore associations between the frequencies of activated and senescent T cells and IMT, and no association was found. Similarly, relations between both variables and hsCRP were explored, and no significant associations were detected (data not shown).

TABLE 3. Multivariate Analysis: HIV-Related Factors Associated With Increased IMT (>p50, 0.42 mm)

	Model 1 (All HIV-Infected Subjects), N = 133			Model 2 (Nonsmokers, Perinatally Infected Subjects), N = 95		
Variable	**OR**	**95% CI**	***P***	**OR**	**95% CI**	***P***
Time with detectable VL (yrs)	1.05	0.95–1.17	0.361	1.08	0.94–1.24	0.275
ART exposure (yrs)	1.04	0.90–1.20	0.590	1.06	0.89–1.26	0.501
PI exposure (yrs)	0.89	0.79–1.01	0.072	0.84	0.72–0.97	0.023
CD4 Nadir (100 cells/mL)	0.82	0.69–0.98	0.033	0.77	0.63–0.95	0.019
Lipodistrophy	0.56	0.21–1.38	0.202	0.68	0.23–2.0	0.494
CD4 counts (cell/mL)	1.04	1.00–1.00	0.383	1.00	0.99–1.39	0.059
CD8 counts (cell/mL)	1.00	1.00–1.00	0.999	0.99	0.99–1.0	0.093

DISCUSSION

Results of our study corroborate the hypothesis that structural changes of the arterial wall appear in the first decades of life during HIV infection, highlighting the need of a prompt diagnosis and treatment of cardiovascular risk factors during childhood. Our findings support a role for the HIV infection itself rather than for HIV-related factors or ART as suggested by other pediatric studies.[7,8] Immune activation and senescence are present since childhood during HIV infection, often related to persistence of detectable VLs, although no relation to subclinical atherosclerosis was detected. To the best of our knowledge, this is the largest study analyzing factors associated with subclinical atherosclerosis in HIV-infected children and adolescents.

Although clinical manifestations of atherosclerosis do not present during childhood, the atherogenic process already begins since earliest stages of life.[21] In our study, we used high-resolution ultrasound measurement of the carotid IMT to assess structural changes of the arterial wall. It is probably the most validated surrogate marker of subclinical atherosclerosis and is considered an independent predictor of adverse cardiovascular events in adults.[22] Several studies have demonstrated increase of IMT in children with known cardiovascular risk factors, such as diabetes, obesity, or metabolic syndrome,[23–26] and many others support the concept that anatomic changes of the arterial wall may improve over time with appropriate intervention.[27–29] Different groups have evaluated atherosclerosis in HIV-infected children by means of IMT

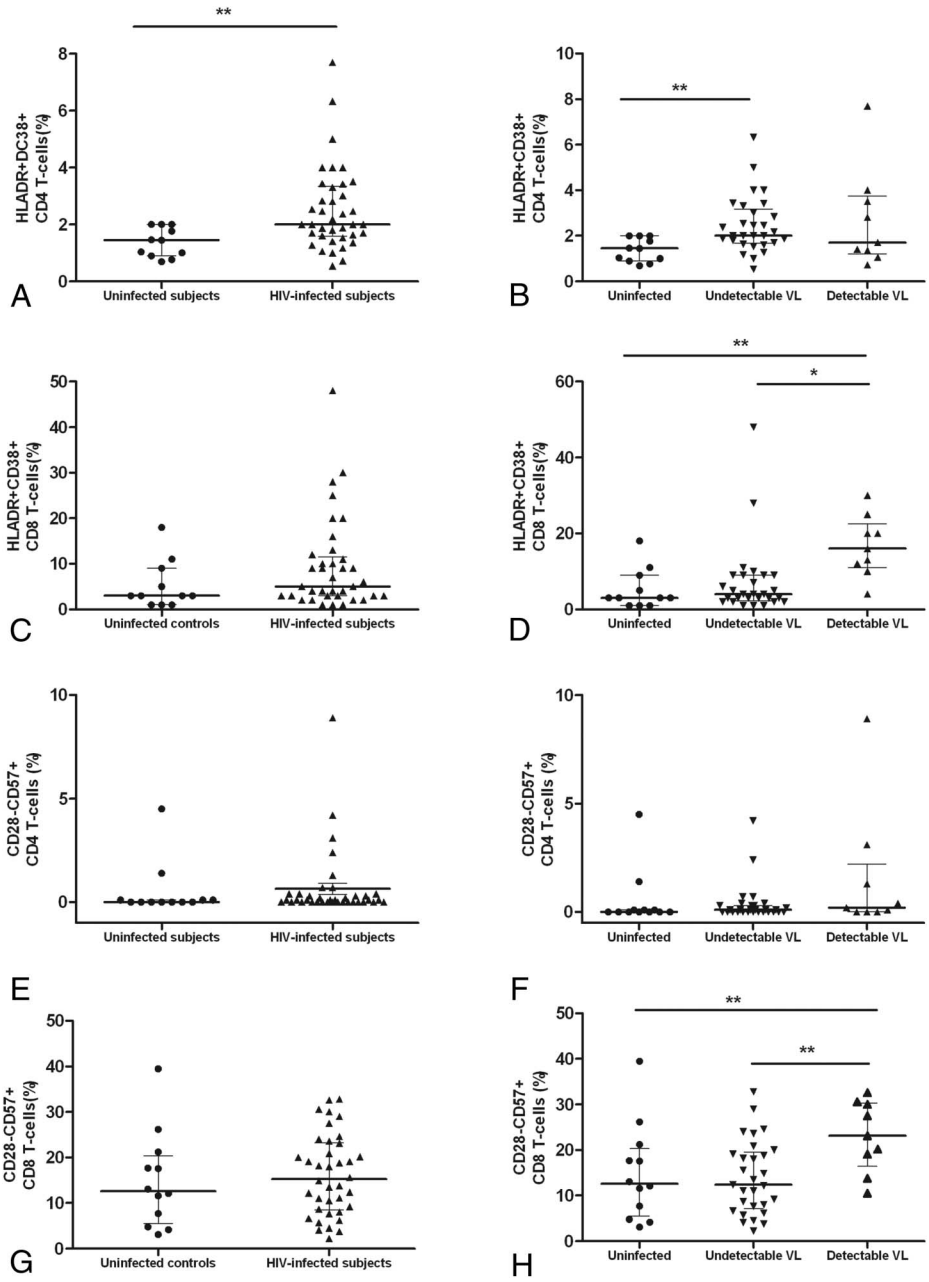

FIGURE 2. Frequencies of activated (HLADR+CD38$^+$) and senescent (CD28-CD57$^+$) CD4 and CD8 T cells (median and IQR) for the study groups. HIV-infected patients had higher levels of HLA-DR$^+$ CD38$^+$ CD4 T cells compared with uninfected subjects (P = 0.016; Fig. 2A). This difference remained significant when the comparison included only those HIV-infected patients on ART who achieved undetectable VL (P = 0.003; Fig. 2B). No difference was found regarding CD4 T-cell activation when comparing those patients that had achieved undetectable VL and those who had not (P= 0.692; Fig. 2B). Although the frequency of activated CD8 T cells was also higher in the HIV-infected group, the difference did not reach statistical significance (P = 0.202; Fig. 2C). However, the frequency of HLA-DR$^+$ CD38$^+$ CD8 T cells was significantly higher in viremic patients compared with aviremic subjects and controls ($P < 0.001$ and P = 0.002, respectively; Fig. 2D). The frequency of CD28$^-$ CD57$^+$ T cells was similar between HIV-uninfected and HIV-infected subjects, both for CD4 and CD8 subsets (P = 0.807, P = 0.495, respectively; Figs. 2E, G). No significant difference in CD28$^-$ CD57$^+$ CD4 T cell was observed when comparing patients with detectable and undetectable VL (Fig. 2F). On the contrary, the percentage of CD28$^-$ CD57$^+$ CD8 T cells was significantly increased among viremic patients compared with uninfected subjects and patients that had achieved undetectable VL (Fig. 2H). P values were computed using the Mann–Whitney and Kruskal–Wallis tests.

measurement.[6,8,9,30–32] Most, but not all these studies showed increased IMT in HIV-infected children compared with uninfected controls, with differences in mean IMT values ranging from 0.02 to 0.15 mm. Although these rather small differences in IMT are of uncertain clinical relevance, we understand that the presence of detectable structural changes of the arterial wall in HIV-infected children is worrisome because these subjects face a long life span to develop CVD. Huge heterogeneity regarding technical aspects of IMT measurement within the studies, inclusion of rather small sample sizes, and the lack of consensus concerning characteristics of the ideal control group may explain some of these divergences among results. The only longitudinal study performed in children published to date has not been able to fully enlighten this complex question to the scientific community[32]. Our findings corroborate the hypothesis that structural changes of the arterial wall appear since childhood in vertically HIV-infected patients, underlining the fact that preventive measures are to be implemented in this high-risk population.

As previously mentioned, the impact of cardiovascular and HIV-related factors on CVD remains controversial as well. Although the effect of antiretroviral therapy on the vasculature has been widely studied in adult population, the existence of a correlation between cardiovascular events and ART exposure (especially PI) remains uncertain.[33–35] Results of some of pediatric studies have suggested an increase of

IMT associated to ART, especially PI or Stavudine exposure.[6,8,31] Nevertheless, results from our study in which cumulative exposure to different antiretroviral regimens was quantified and included in the multivariate analysis, show no relation between PI or ART exposure and subclinical atherosclerosis. Moreover, PI exposure seems to be more likely a protective factor than a proatherogenic one in this large cohort of vertically HIV-infected patients. Among other HIV-related variables, CD4 T-cell count and CD4 nadir have been proposed to be the most robust risk factor for increased IMT in the adult population.[36,37] Similarly, CD4 nadir has shown to be associated with thicker IMT in our study, supporting the hypothesis that immunity might play a strong role on the atherogenic process during HIV infection.

These data support the wide body of evidence suggesting that HIV itself plays a role in the development of atherosclerosis.[38–41] Among underlying mechanisms, chronic inflammation and immune activation have been proposed to be at least partially responsible of the atherogenic process.[6] Recent studies in adults have shown that HIV-associated T-cell changes are associated with subclinical carotid artery abnormalities; higher frequencies of activated CD4 and CD8 T cells and senescent CD8 T cells are associated with an increased prevalence of carotid artery lesions, although no relation to IMT could be demonstrated.[5] To date, very few studies have focused on the effect that increased activation/senescence may have on premature clinical aging in children. In our study, higher frequencies of activated CD4 T cells were found in HIV-infected children when compared with uninfected controls. Frequencies of activated and senescent CD8 T cells were markedly increased only in those patients with detectable VL, along with findings from previous studies in children,[10,11] pointing out the important effect of achieving viral suppression on the CD8 T-cell subsets. However, no relation could be established with IMT thickness. Although the minimal dispersion of IMT values in our study population may have underpowered the analyses to detect statistically significant associations with markers of T-cell activation, these findings are consistent with the recently published results from other pediatric studies, which suggest that T-cell activation is not associated to disease progression in vertically HIV-infected children.[12,42] Levels of T-cell activation were rather low in our ART-treated group. A possible explanation for that finding is the fact that CMV coinfection may not be as prevalent in HIV-infected children as it is in adults. CMV seems to act synergistically to increase immune activation during HIV infection because both viruses coexist in most patients and in fact, CMV infection has been linked in HIV-infected adults to T-cell activation and atherosclerosis.[43,44] Unfortunately, in our study, CMV serostatus of the participating children was not determined, and thus, effect of CMV infection could not be isolated. Whether there is a real association between HIV-induced T-cell activation and senescence and IMT remains to be answered.

To minimize the weight of classical cardiovascular risk factors, controls were enrolled aiming to achieve a group with similar age, sex, ethnicity, and BMI. However, the nature of the control group might be a limitation of the study. Uninfected ART exposed children and siblings have been proposed as the optimal control group, as socioeconomical background determines many cardiovascular risk factors. Nevertheless, it is not well known the effect that intrautero exposure to ART might have on cardiovascular parameters, and thus, recruitment of the ideal control group remains to be a challenge. In our study, no differences were found regarding absolute values of BMI between groups; however, HIV-infected children showed a statistically significant lower z score adjusted BMI. We understand that this fact could have led to underestimate the difference in IMT values between both cohorts because there is a positive correlation between IMT and BMI and provides with an additional value to the difference found.

In conclusion, we herein corroborate in a large cohort of patients that structural changes of the vasculature present early in HIV-infected subjects. CD4 nadir but not ART seems to be related to the described increased in cardiovascular risk. Most studies showing increased risk of cardiovascular events during HIV infection include adults of age around 50 years old who have been living along with the virus for a median of 3–8 years.[34,45] By the time they grow that old, our vertically HIV-infected children will have been living with the infection for a period of 5–10 times longer. Thus, these patients should be carefully monitored for the prompt detection and early treatment of noninfectious disorders, and strategies are urgent to be defined for the prevention of CVD in this unique population. Long-term longitudinal follow-up of the patients included in this study is ongoing for a better understanding of the vascular changes and to ascertain the contribution of different HIV-related factors to cardiovascular risk.

SUMMARY

Associations between clinical factors, markers of inflammation, immune activation/senescence, and carotid IMT are analyzed in 150 HIV-infected children and 150 HIV-uninfected controls.

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CAPÍTULO 2

Marcadores inflamatorios y cardiovasculares y enfermedad vascular subclínica.

JUSTIFICACIÓN Y OBJETIVOS

Numerosos estudios demuestran la presencia de enfermedad vascular subclínica en los adultos infectados por el VIH, y ya existen evidencias de que el proceso arterosclerótico comienza en presencia del VIH en edades tempranas de la vida. Los pacientes infectados por el VIH por transmisión vertical son una población única considerada de alto riesgo para el desarrollo de complicaciones secundarias, asociadas a la infección pero también a la exposición crónica al tratamiento antirretroviral. Pese a la creciente preocupación por el aumento de RCV descrito en la población infectada por el VIH, no se han establecido hasta el momento medidas preventivas y de tratamiento específicas. Se han estudiado numerosos biomarcadores con el objetivo de, por un lado, contribuir a explicar la fisiopatología del proceso aterosclerótico, y por otro, lograr la identificación de sujetos en situación de mayor RCV. Establecer marcadores válidos de RCV y desarrollar herramientas que permitan identificar a los sujetos que padecen mayor probabilidad de sufrir eventos cardiovasculares desfavorables se presenta como uno de los grandes desafíos clínicos de la infección por el VIH. Además, desde las consultas de pediatría se tiene la oportunidad de intervenir en las fases iniciales del proceso de remodelamiento arterial. Los estudios realizados hasta la fecha en población pediátrica orientados a evaluar diferentes biomarcadores inflamatorios y de RCV no han obtenido resultados concluyentes.

Los objetivos de este capítulo son:

- Estudiar una serie de marcadores inflamatorios, hemostáticos y endoteliales, en un subgrupo de niños y adolescentes infectados por el VIH con buena situación inmunológica y ausencia de alteraciones metabólicas, en comparación con controles sanos no VIH.
- Estudiar los niveles del marcador de activación monocitaria CD14 soluble, considerado un maracdor indirecto de translocación bacteriana.
- Analizar la asociación de estos biomarcadores con un marcador subrogado de RCV, como es el GIM.
- Analizar los factores asociados a la infeccióon por el VIH relacionados con elevación de los biomarcadores.

CONCLUSIONES

- Un total de 64 niños y adolescentes infectados por el VIH y 30 controles se incluyeron en este subestudio. Un 64% eran mujeres, y la edad media fue de 14,1 ± 5 años. Los pacientes incluidos en el estudio se encontraban todos en tratamiento, presentaban muy buena situación clínica y un excelente control metabólico. No se encontraron diferencias significativas entre los grupos en cuanto a edad, sexo, IMC o medidas antropométricas, ni tampoco en cuanto a perfil lipídico, con tan solo una mínima elevación de triglicéridos en el grupo VIH.
- Los valores de los biomarcadores estudiados (PCR IL-6, mieloperoxidasa, MCP-1, P-selectina, y factor activador tisular del plasminógeno) fueron similares en ambos grupos, a excepción de sVCAM, elevada en el grupo de pacientes infectados por el VIH.
- No se identificaron factores asociados a la infección por el VIH relacionados con este incremento descrito de sVCAM.
- Ninguno de los biomarcadores analizados se asoció de manera significativa con el grosor íntima-medio carotideo.
- Los pacientes infectados por el VIH presentaban elevación del marcador de activación monocitaria sCD14. Los valores de sCD14 se correlacionaron positivamente con los niveles de PCR, y negativamente con el nadir de CD4 (p<0,05), pero no con un aumento del el grosor íntima-medio carotideo.
- En ausencia de otras medidas específicas de prevención de RCV, un estricto control de las alteraciones metabólicas secundarias al tratamiento antirretroviral debe ser considerado una prioridad en el manejo de los niños y adolescentes infectados por el VIH.

Cardiovascular Biomarkers in Vertically HIV-Infected Children Without Metabolic Abnormalities

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ABSTRACT

Early cardiovascular disease is a major concern for ART-suppressed vertically HIV-infected children; however, evidence is lacking regarding specific preventive measures. In this study, a complete panel of biomarkers was determined together with carotid intima-media thickness (IMT), in a cohort of 64 HIV-infected children and 30 controls. Mean age of participants was 14.1 ± 5 years. HIV-infected patients showed normal lipid profile, with only slightly higher triglycerides, and no differences between groups were found regarding IMT. HIV-infected

patients displayed higher levels of soluble CD14 (sCD14) and soluble vascular cell adhesion molecule-1 (sVCAM) (all $p<0.05$). However, levels of C-reactive protein, interleukin-6, myeloperoxidase, monocyte chemoattractant protein -1, P-selectin and tissue plasminogen activator were similar between groups. Vertically HIV-infected subjects on ART with no significant metabolic disturbances displayed increased sCD14 and sVCAM but not up-regulation of proinflammatory pathways. Larger studies are warranted to assess the impact of a strict metabolic control on cardiovascular risk and to define specific cardiovascular disease preventive strategies in this population.

Keywords: HIV; children and adolescents; cardiovascular risk; IMT; CRP; cardiovascular biomarkers; sCD14.

Cardiovascular Biomarkers in Vertically HIV-Infected Children Without Metabolic Abnormalities

Introduction

HIV infection is associated with vascular dysfunction and adverse cardiovascular outcomes, likely driven by up-regulation of inflammatory pathways, accumulation of classical cardiovascular risk factors and ART-related metabolic disturbances [1]. The scope of cardiovascular disease may become even greater in years to come in the special population of vertically HIV-infected subjects, with lifelong exposure to inflammation and metabolic abnormalities. Indeed, previous studies suggest that vertically HIV-infected adolescents already exhibit subclinical atherosclerosis [2-4].

Although extensively analyzed, the pathophysiology of atherosclerosis within HIV infection remains to be entirely elucidated. HIV-infection is characterized by increased concentrations of proinflammatory cytokines, such as C-reactive protein (CRP) and interleukin (IL)-6, even under viral suppression [5]. Importantly, some of these markers strongly predict cardiovascular disease and mortality [6, 7]. Soluble CD14 (sCD14), a marker of monocyte activation used as an indirect measure of microbial translocation, has been associated to disease progression and mortality, although its relation to endothelial dysfunction remains controversial [8, 9]. Most of the evidence in this field comes from studies performed in adults, and data supporting increased levels of proinflammatory cytokines during childhood are controversial [2-4, 10, 11].

Unraveling the proatherogenic pathways up-regulated in vertically HIV-infected individuals remains critical in order to design strategies for cardiovascular disease prevention in this particular population. In this scenario, we assessed relations between intima-media thickness (IMT) and proinflammatory cytokines [IL-6, CRP, myeloperoxidase (MPO)], tissue plasminogen activator (tPA), adhesion molecules [Soluble vascular cell adhesion molecule-1 (sVCAM-1), and

pSelectine] and sCD14, in a cohort of ART-treated HIV-infected children and adolescents and healthy controls.

Methods

Study design and eligibility criteria

We performed a sub-study within an ongoing longitudinal, observational study evaluating cardiovascular risk in HIV-infected children and healthy controls [12]. Participants with available plasma samples were included in this analysis. Exclusion criteria included acute or opportunistic infections, family history of premature CVD, chronic conditions and use of medications other than ART.

The study was reviewed and approved by the Ethics Committee and Clinical Research of the participating Hospitals. All participants, parents or legal guardians and children over twelve years, gave written informed consent to take part in the study.

Clinical assessments

Data were collected prospectively. Weight, height, Body Mass Index (BMI) and hypertension were adjusted using z score according to the age and gender [13,14]. Immunovirological details and previous ART history were collected from the Spanish Paediatric HIV Network (CoRISpe) database [15].

Laboratory assays

Fasting blood samples were drawn for real-time measurements of glucose levels and lipid profile (total cholesterol, high-density lipoprotein cholesterol [HDLc], low-density lipoprotein cholesterol [LDLc]), determined using standard enzymatic methods. Plasma HIV-1 viral load (VL) was quantified using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Inc, Branchburg, NJ) with a detection limit of 50 copies/mm^3. CD4 T-cell counts were concomitantly measured with standard flow cytometric methods.

Levels of sCD40L, IL-6, MCP-1, P-selectin, MPO, sVCAM and t-PA were measured from frozen samples processed and stored at the Pediatric HIV Biobank [16], using a Human Cardiovascular FlowCytomix Multiplex kit (eBioscience, San Diego, California). High sensitivity CRP and sCD14 were analyzed with a commercial ELISA kit (eBioscience, Inc, San Diego, CA and R&D Systems, Minneapolis, MN, respectively).

Carotid artery ultrasound

IMT measurements were made bilaterally at the common carotid artery (1-2 cm proximal to the bulb) as previously described [12], by a trained technician, using a CX50 ultrasonographic portable equipment, and specific IMT detection software QLab (Philips Medical Systems, Inc., Eindhoven, The Netherlands). Images were read by an experienced cardiologist, blind to HIV status.

Data analyses

Means for variables with a normal distribution were compared using the Student's t-test, and non-parametric variables were examined using the Mann-Whitney and Kruskal-Wallis tests. Statistical power of the study, using mean difference in CRP as a reference and an alpha of 0.05, was 0.95. Differences in biomarkers between groups were adjusted by sex and age in a multivariate logistic regression analysis. Multivariate linear regression analyses were built to further analyze the associations between biomarkers and HIV-related variables, using for the different models each biomarker as the dependent variable. The null hypothesis was rejected by a type I error <0.05. Statistical analyses were performed using Stata v. 12.0 (StataCorp LP College Station, Texas, USA).

Results

Study population and between group comparison of clinical characteristics

A total of 64 HIV-infected children and adolescents and 30 healthy controls were included, with a mean age of 14.1 ± 5.0 years. Main characteristics of both cohorts are summarized in table 1.

HIV-infected and uninfected subjects showed similar age, gender, BMI, blood pressure and frequency of hypertension. Of note, there were no significant differences between groups regarding levels of total cholesterol, HDLc or LDLc, and only triglycerides were mildly elevated. Although median IMT was slightly higher in the group of HIV-infected patients, the difference between groups did not reach statistical significance (Table 1).

Table 1. Main characteristics of both cohorts

	HIV-infected subjects (N=64)	Control subjects (N=30)	p
Demographic data			
Age (years)	14.2 (4.85)	14.1 (5.52)	0.635
Female, N (%)	44 (68)	16 (54)	0.147
Caucasians, %	62.5	83.3	0.426
Cardiovascular risk factors			
Systolic blood pressure (mmHg)	107.2 (10.5)	106.3 (15)	0.747
Diastolic blood pressure (mmHg)	60.6 (8.1)	61.5 (7.9)	0.619
Hypertension, %	3.6	3.2	0.922
Smokers, %	14.3	6.7	0.288
Weight (Kg)	45.8 (17.3)	48.9 (17.2)	0.412
Height (cm)	150.5 (19.9)	153.6 (25.5)	0.521
BMI (kg/m²)	19.4 (4.1)	19.8 (2.8)	0.594
z score BMI*	0.00 (-1 - 0.5)	0.25 (-0.5- 1)	0.152
Waist (cm)	68.8 (10.7)	69.3 (9.9)	0.415
Hip (cm)	80.4 (13.2)	81.8 (12.1)	0.317
Waist-to-hip ratio	0.86 (0.1)	0.85 (0.8)	0.671
Glycemia and lipid profile			
Glucose (mg/dL)	86.2 (9.3)	83.3 (11)	0.130
Total cholesterol (mg/dL)	172.6 (40.3)	170.7 (44.7)	0.543
>200mg/dL	14 (21.8)	5 (16.6)	0.558
HDLc (mg/dL)	50.5 (12.9)	57.3 (15.2)	0.068
<50mg/dL, N (%)	27 (42)	11 (36.6)	0.611
LDLc (mg/dL)	97.9 (29.2)	96.9 (42.3)	0.299
Total cholesterol/HDLc	3.6 (1)	3.1 (1.2)	0.299
Triglycerides (mg/dL)*	98 (72 - 150)	76.5 (57 - 97)	0.002
>150mg/dL, N (%)	17(26.6)	1(3.3)	0.008
Plasma biomarkers			
C Reactive protein (mg/L)*	0.15 (0.09 - 0.61)	0.07 (0.02 - 0.29)	0.188
sCD14 (microg/mL) *	1.93 (1.57-2.28)	1.41 (1.20-1.65)	<0.001
Il-6 (pg/mL) *	0 (0 - 2.1)	0.52 (0 - 3.6)	0.511
sCD40L (pg/mL) *	781 (428 - 2261	1101 (798 - 1853)	0.365
MCP-1 (pg/mL) *	292 (139 - 374)	240 (218 - 395)	0.218

sP-selectin (ng/mL) *	2905 (2383 - 3322)	3603 (2238 - 4223)	0.293
MPO (ng/mL) *	51.5 (43.5 - 66.3)	47.8 (41 - 58)	0.255
sVCAM (microg/mL) *	958 (23 - 1129)	535 (6.3- 1129)	0.001
t-PA (pg/mL) *	2501 (1820 - 3005)	2229 (1322 - 2812)	0.043
Intima-media thickness (mm)	0.433 (0.02)	0.427 (0.02)	0.188
HIV-related parameters			
On ART (%)	100		
Receiving NNRTI, N (%)	28 (43.7)		
Mean (SD) duration of NNRTI therapy, y	3.16 (2.52)		
Receiving PI, N (%)	37 (57.8)		
Mean (SD) duration of PI therapy,y	3.10 (2.27)		
Lopinavir/ritonavir containing regimen, N (%)	25 (67.6)		
Atazanavir or Darunavir containing regimen, N (%)	12 (32.4)		
Vertical transmission (%)	100		
HVC coinfection (%)	4		
Viral Load <50 cop/mL (%)	82.8		
CD4 count (cell/uL)*	777 (529- 1099)		
Nadir CD4 (cell/uL)*	363 (214-576)		

Results are expressed as mean (standard deviation), except otherwise specified.
**Expressed as median (IQR)*
Hypertension: Systolic blood pressure >p95 of reference values
Abbreviations; BMI, body mass index [calculated as weight in kilograms divided by the square of height in meters]; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; sCD14, soluble CD14; Il-6, Interleukin 6; sCD40L, soluble CD40 ligand; MCP-1, monocyte chemotactic protein-1, sP-selectin, soluble P selectin; MPO, myeloperoxidase; sVCAM, soluble vascular cell adhesion molecule-1; t-PA, tissue plasminogen activator; ART: antiretroviral treatment; PI, protease inhibitor; NNRT, non-nucleoside analogue reverse-transcriptase inhibitors; NRTI, nucleoside analogue reverse-transcriptase inhibitors.

Between group comparison of inflammatory, endothelial and hemostatic markers

HIV-infected patients showed increased levels of sCD14, tPA and sVCAM, but no statistically significant differences were found in other biomarkers (Table 1). When results were adjusted by age and gender, only differences in sCD14 and sVCAM remained statistically significant (β= 0.49, p<0.001 and β= 424, p=0.001, respectively). As HIV-infected subjects showed higher tryglicerides and lower HDL levels, we also adjusted the multivariate models by these two variables, and the differences in both biomarkers remained statistically significant.

In order to assess the contribution of HIV suppression to the observed differences in biomarkers, comparisons were restricted to the 53 patients with undetectable VL, and results did not vary (data not shown).

Associations between elevated biomarkers and HIV-related variables

We first compared the concentrations of plasma biomarkers between patients with detectable and undetectable VL, and no statistically significant differences were detected (data not shown). After the multivariate analysis including age, sex, current VL and cumulative ART exposure, a lower CD4 nadir predicted increased levels of sCD14 (per 100cells-increment, β= -0.052526, p=0.045), adjusted by current CD4 counts. Levels of sCD14 weakly correlated with CRP (Spermans' Rho: 0.25, p=0.04), and the association was stronger when only suppressed patients were included in the analysis (Spermans' Rho: 0.42, p<0.01).

No other significant associations were detected among biomarkers and with HIV-related variables, and none of the biomarkers correlated with IMT measurements.

Discussion

In this study we evaluated carotid atherosclerosis and an extensive panel of biomarkers involved in inflammatory, endothelial, and hemostatic pathways in a cohort of vertically ART-treated HIV-infected subjects and healthy controls. No differences in lipids, BMI, visceral obesity or IMT were found between groups. In this scenario of lack of relevant ART mediated metabolic disorders and good disease control we found similar levels of most biomarkers between both groups, with the exception of sCD14 and sVCAM.

Most studies – including our previous results, agree that vertically HIV-infected children already present subclinical atherosclerosis [2-4, 12]. However, results regarding inflammatory biomarkers are far from being consistent, and there is only one study describing a link between CRP levels and IMT in children [4]. The use of different biomarker panels and rather small sample sizes may account for the irreproducibility of results. Although a good metabolic or

viremic control was not required for participation, HIV-infected subjects in our study showed only mild hypertriglyceridemia and were mostly suppressed. In this context, only levels of sVCAM, a molecular marker of atherosclerosis [17], were significantly increased. These results are consistent with the recently published study by Miller et al., which supports a role for active virus replication and lipid abnormalities in the elevation of cardiovascular biomarkers in HIV-infected children [11]. If further confirmed, these results are encouraging, since they suggest that proatherogenic mechanisms associated to HIV-infection might be attenuated whenever ART is established and deleterious ART-related metabolic effects are controlled. Most of the previous studies in HIV-infected children include patients displaying severe ART-related metabolic disturbances, and thus, the effect of the virus itself on cardiovascular biomarkers may be difficult to isolate. The fact that most subjects are on treatment may have contributed as well to our findings, as ART has shown to decrease –although not completely normalize– inflammation [18,19]. Although we have previously described a significant increase of IMT in our cohort of HIV-infected children and adolescents [12], the difference in the subgroup of patients included in this cross-sectional analysis was non-significant, probably due to a lower statistical power related to sample size.

In this study, sCD14 concentrations were increased among HIV-infected subjects despite viral suppression, and positively correlated with levels of CRP. These findings are consistent with previous studies supporting the hypothesis that ongoing microbial translocation during treated HIV infection has a dominant role in fueling inflammation in vertically HIV-infected patients [20], and may be contributing to the risk of future cardiovascular disease.

We did not detect any significant association between the different biomarkers and the presence of detectable VL, and from all other HIV-related variables, only a lower nadir CD4 predicted increased levels of sCD14. The negative correlation between nadir CD4 and sCD14 suggests that starting therapy early might be particularly relevant for this population, in order to reach better GALT restoration. The frequency of viral load peaks during adolescence precludes us from drawing conclusions from this particular finding.

ART-treated vertically HIV-infected subjects in our study showed no significant metabolic disturbances. In this scenario, no up-regulation of proinflammatory pathways was found, except for higher levels of sCD14 and sVCAM. Larger studies are warranted in order to characterize the impact of a strict control of metabolic alterations on cardiovascular risk and to define specific preventive strategies in this special population.

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CAPÍTULO 3

El cociente CD4/CD8 como marcador de activación y senescencia del sistema inmune en los niños y adolescentes infectados por el VIH.

JUSTIFICACIÓN Y OBJETIVOS

Pese a alcanzar la supresión viral y la restauración inmune con el TAR, la activación inmune ha demostrado ser un predictor robusto de progresión de la enfermedad por el VIH, y uno de los mecanismos subyacentes al fenómeno de inmunosenescencia, envejecimiento prematuro y eventos clínicos desfavorables descritos en los pacientes infectados por el VIH, que parecen ser los responsables de que la esperanza de vida de los pacientes infectados no alcance las cifras de la población general. Los efectos de esta activación crónica del sistema inmune y de la inmunosenescencia serán probablemente más graves en el colectivo de pacientes de transmisión vertical, cuyo sistema inmune se desarrolla en presencia del virus, y con muchos más años por delante para desarrollar complicaciones asociadas a la infección. El control de la inflamación y activación inmunes se presenta como el gran desafío clínico en el manejo de la infección por el VIH en la era post-TAR. El desarrollo de herramientas que permitan identificar a los individuos con mayor riesgo de desarrollar complicaciones asociadas al envejecimiento prematuro, es por tanto una necesidad.

Estudios realizados en población general sugieren que la inversión del cociente CD4/CD8, característica de la infección por el VIH sin tratamiento y también asociada a la infección por CMV, es un marcador pronóstico de inmunosenescencia, y un predictor independiente de mortalidad. Aunque se ha descrito que muchos pacientes no llegan a normalizar el cociente CD4/CD8 tras el inicio del TAR, la dinámica del cociente no ha sido bien caracterizada en los pacientes en tratamiento.

Los objetivos de este capítulo son:

- Determinar con qué frecuencia el cociente CD4/CD8 se encuentra invertido en niños y adolescentes infectados por el VIH pese a lograr una buena recuperación inmunológica con el TAR.
- Determinar factores clínicos asociados a la inversión del cociente.
- Evaluar la utilidad del cociente CD4/CD8 como marcador de los fenómenos de activación y senescencia inmune.

CONCLUSIONES

- En este estudio transversal se incluyeron un total de 37 niños y adolescentes con infección por el VIH. La mediana de edad fue de 16,4 ± 3,8 años. Todos los pacientes se encontraban en tratamiento, y un 76% presentaba CV indetectable (<50 copias/mL).
- La mediana de CD4 fue de 783 cel/mm^3, la mediana del nadir de CD4 de 310 cel/mm^3, y la mediana del cociente de CD4/CD8 1,24. Se observó inversión del cociente CD4/CD8 (<1) en 16 pacientes (43,2%).
- Se encontró una correlación negativa entre el cociente CD4/CD8 y los tiempos de exposición a antirretrovirales y tiempo de supresión viral, así como con la edad. No se encontró asociación con el nadir de linfocitos T CD4.
- Se observó una correlación negativa fuerte entre el cociente CD4/CD8 y la frecuencia de linfocitos T CD8 que expresaban marcadores de activación (HLADR+CD38+), senescencia (CD28-CD57+), y agotamiento inmunológico (HLADR+PD-1+).
- La inversión del cociente se asoció a disminución en la proporción de linfocitos T CD4 y CD8 naïve (54,0% vs. 61% y 31,5% vs. 48,6%, respectivamente) y un incremento en el porcentaje de células T CD8 de memoria efectora (10,2% vs. 5,2%) (p<0,05 para todas las comparaciones).
- Tras ajustar por edad, recuento total de CD4, nadir de CD4, tiempo de TAR y tiempo en supresión viral, el cociente CD4/CD8 se asoció de forma independiente con la frecuencia de linfocitos T CD8 con fenotipo activado (B=-6,1, p=0,024), senescente (B=-5,5, p=0,016) y de agotamiento inmunológico (B=-15,5, p=0,001).
- Estos hallazgos sostienen la hipótesis de que la persistencia de un cociente CD4/CD8 invertido pese a una buena recuperación inmunológica con el TAR podría ser un marcador que reflejase la existencia de un estado suyacente de activación y senescencia inmunes, y que por tanto identificase a los sujetos con mayor riesgo de sufrir complicaciones asociadas al envejecimiento prematuro. Este hecho podría tener importantes implicaciones diagnósticas y terapéuticas.

Research Letter

AIDS 2013, **27**:1513–1519

The CD4/CD8 ratio as a marker T-cell activation, senescence and activation/exhaustion in treated HIV-infected children and young adults

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We explored the associations of the CD4/CD8 ratio with markers of immunoactivation, immunosenescence and T-cell subsets, in 37 vertically HIV-infected children and adolescents. CD4/CD8 ratio inversion was associated with higher frequencies of activated, senescent and activated/exhausted $CD4^+$and $CD8^+$ T-cells, and a skewed T-cell phenotype from naive toward effector memory which persisted after the multivariate analysis. Thus, the CD4/CD8 ratio may identify patients with higher immunoactivation despite ART.

Immunoactivation has demonstrated to be a strong predictor of disease progression in HIV-infection, and one of the underlying causes leading to immunosenescence, premature aging and adverse outcomes among patients with access to modern ART regimens [1]. The effects of chronic immunoactivation and immunosenescence are likely to be more pernicious in vertically HIV-infected individuals, since their immune system coevolves since birth with the virus.

Inversion of the CD4/CD8 ratio (<1), a hallmark of untreated HIV infection, is a surrogate marker of immunosenescence and independently predicts all-cause mortality in the general population [2–5]. Although a failure to normalize the CD4/CD8 ratio is commonly observed HIV-infected patients after starting ART, its biological significance and clinical relevance remain unknown.

We hypothesized that a low CD4/CD8 ratio despite ART could be a predictor of increased immunoactivation and immunosenescence in vertically-HIV infected children and adolescents.

HIV-infected children and adolescents on stable ART were enrolled in a cross-sectional multicenter study. Exclusion criteria included acute or opportunistic infections and chronic inflammatory diseases. The study was approved by the Ethics Committee and all parents or legal guardians and children over 12 years gave written informed consent. Plasma viral load was measured using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Inc., Branchburg, New Jersey, USA) with a detection limit of 50 copies/μl. $CD4^+$ and $CD8^+$ T-cell counts were determined using a Gallios flow cytometer and data analyzed using Kaluza software (Beckman Coulter, Fullerton, California, USA). T-cell activation was characterized by $HLADR^+$ $CD38^+$ expression, senescence by $CD57^+$ $CD28^-$, and activation/exhaustion by $HLADR^+PD\text{-}1^+$, in $CD4^+$ and $CD8^+$ T-cells. T-cell subsets were defined as follows: naive ($CD45RA^+CD27^+$), central memory ($CD45RO^+$ $CD27^+$) and effector memory ($CD45RO^+CD27^-$).

Patients were classified according to the presence of a normal (≥1) or an inverted (<1) CD4/CD8 ratio. Mann–Whitney tests were used for independent two-group comparisons and the Spearman correlation coefficient to analyze the correlation between continuous variables. Independent associations between $CD4^+$ and $CD8^+$ T-cells expressing markers of activation, senescence and activation/exhaustion (as dependent variables), with a limited subset of independent variables (including the CD4/CD8 ratio) were explored by a series of multivariate linear regression models. The crude correlation coefficients between the percentage of $CD4^+$ and $CD8^+$ T-cells with phenotypes of activation, senescence and activation/exhaustion and the CD4/CD8 ratio were calculated, followed by consecutive multivariate analyses adjusting by age, time on viral suppression, HIV-1 RNA less than 50 copies/μl, $CD4^+$ T-cell nadir and accumulated ART exposure for all dependent variables. Statistical analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, Illinois, USA).

Thirty-seven vertically HIV-infected children and adolescents were included, with a mean age of 16.4 ± 3.8 years. Twenty-eight patients (75.7%) presented viral load less than 50 copies/μl. Median accumulated exposure to ART was 13.8 years (8.6–15.5), and mean time on viral suppression 4.8 years (1.8–7.3). Median CD4 nadir was 310 cells/μl, median CD4 cell count 783 cells/μl and median CD4/CD8 ratio 1.24. Sixteen (43.2%) patients showed CD4/CD8 ratio inversion (<1).

Main correlations between the CD4/CD8 ratio and clinical and immunological variables are shown in Fig. 1. The CD4/CD8 ratio positively correlated with $CD4^+$ T-cell count, accumulated ART exposure and time on viral suppression. In contrast, the CD4/CD8 ratio inversely correlated with age and $CD8^+$ T-cell count. No significant correlation was observed with the $CD4^+$

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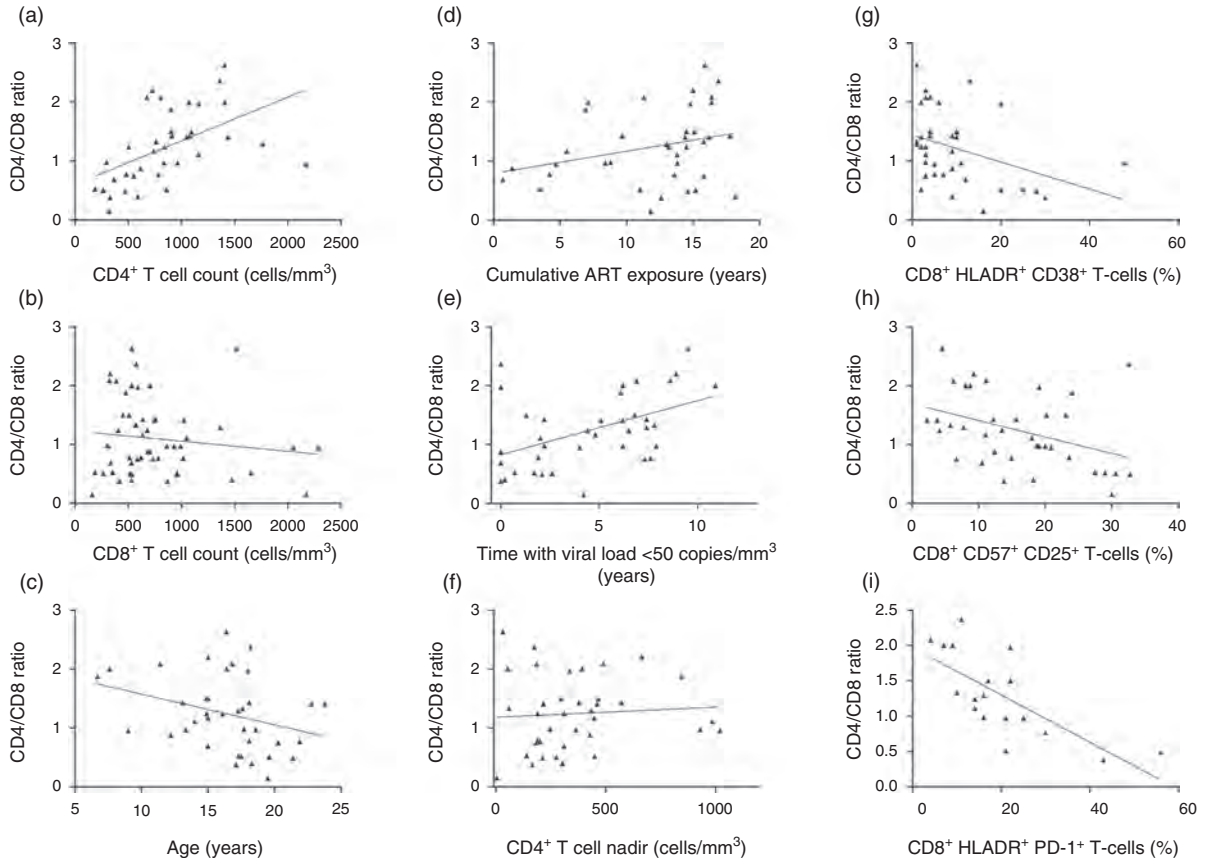

Fig. 1. CD4/CD8 ratio relations with age, therapy, immunovirological variables, CD8$^+$ T-cell activation, senescence and activation/exhaustion. The CD4/CD8 ratio was positively correlated with CD4$^+$ T-cell count ($r = 0.614$, $P < 0.001$) (a), and inversely correlated with CD8$^+$ T-cell count ($r = -0.494$, $P = 0.002$) (b) and age ($r = -0.342$, $P = 0.038$) (c). There was a positive correlation between CD4/CD8 ratio and cumulative antiretroviral therapy (ART) exposure ($r = 0.339$, $P = 0.040$) (d) and time with undetectable viral load ($r = 0.457$, $P = 0.004$) (e). No significant correlation was observed with the CD4$^+$ T-cell nadir ($r = 0.164$, $P = 0.332$) (f). CD4/CD8 ratio was negatively correlated with the percentages of activated CD8$^+$ T-cells ($r = -0.414$, $P = 0.012$) (g) and senescent CD8$^+$ T-cells ($r = -0.389$, $P = 0.017$) (h). CD4/CD8 ratio strongly correlated with CD8$^+$ T-cells expressing the activated/exhausted phenotype ($r = -0.752$, $P < 0.001$).

T-cell nadir. The CD4/CD8 ratio negatively correlated with the percentages of activated and senescent CD4$^+$ and CD8$^+$ T-cells, and a strong negative correlation with T-cells expressing the activated/exhausted phenotype was observed.

Compared with those with normal CD4/CD8 ratio, patients with an inverted CD4/CD8 ratio showed increased CD4$^+$ and CD8$^+$ T-cell activation (2.8 vs. 1.8% and 10.3 vs. 3.4%, respectively), senescence (0.2 vs. 0.04% and 19.6 vs. 11.0%, respectively) and activation/exhaustion (5.0 vs. 2.2% and 25.0 vs. 14%, respectively) (all comparisons $P < 0.05$, except for CD4$^+$ T-cell senescence, $P = 0.095$). Regarding T-cell subpopulations, CD4/CD8 ratio inversion was associated with lower frequencies of naive CD4$^+$ and CD8$^+$ T-cells (54.0 vs. 61% and 31.5 vs. 48.6%, respectively) and increased effector memory CD8$^+$ T-cells (10.2 vs. 5.2%) (all comparisons $P < 0.05$).

After consecutive linear regression models, adjusting by age, time on viral suppression, HIV-1 RNA less than 50 copies/μl, CD4$^+$ T-cell nadir and accumulated ART exposure, the CD4/CD8 ratio remained independently associated with CD4$^+$ T-cell activation ($B = -1.1$, $P = 0.003$), CD8$^+$ T-cell activation ($B = -6.1$, $P = 0.024$), CD8$^+$ T-cell senescence ($B = -5.5$, $P = 0.016$) and CD8$^+$ T-cell activation/exhaustion ($B = -15.5$, $P = 0.001$), but not with CD4$^+$ T-cell senescence ($B = -0.786$, $P = 0.074$) or CD4$^+$ T-cell activation/exhaustion ($B = -2.4$, $P = 0.343$).

In our study in vertically HIV-infected children and adolescents, we found an independent inverse association between the CD4/CD8 ratio and the frequencies of T-cells expressing markers of activation, senescence and activation/exhaustion. Inversion of the CD4/CD8 ratio was also associated with lower levels of naïve CD4$^+$ and CD8$^+$ T-cells, supporting the hypothesis that an inverted

CD4/CD8 ratio despite peripheral $CD4^+$ T-cell restoration may reflect an underlying immunoactivation and immunosenescence.

In the general population, inversion of the CD4/CD8 ratio is considered a surrogate marker of the so-called immune risk profile [5], characterized by an increase in the number of $CD8^+CD28^-$ T-cells and associated with cytomegalovirus (CMV)-specific T-cells [2]. Moreover, the CD4/CD8 ratio has directly been associated with all-cause mortality in two European cohorts [4,6,7]. Many of these immunological alterations observed in the elderly are similar to those that take place in HIV-infected patients [8], such as expansion of CMV-specific T-cells [9], decreased levels of naïve $CD8^+$ T-cells and increased memory $CD8^+$ T-cells [8,10–12]. Thus, the elderly show lower CD4/CD8 ratio than younger patients [13], and on the contrary, children display higher CD4/CD8 ratio than adults [14]. This consideration must be kept in mind when interpreting our results given the wide range of age of our study population (6–23 years), and accordingly age was considered as a potential confounder in multivariate analyses.

Few studies have addressed the significance of the CD4/CD8 ratio in HIV infection. Before the introduction of the highly active ART in 1996, the CD4/CD8 ratio was identified as a predictor of disease progression [15]. More recently, a low CD4/CD8 ratio at initiation of ART has been associated with the prevalence and volume of coronary plaques [16]. We have previously reported an association between the CD4/CD8 ratio and immunoactivation in HIV-infected adults with long-term viral suppression [17]. Immunoactivation and immunosenescence are also associated with other surrogate markers of coronary events in HIV-infected patients, such as carotid intima-media thickness [18,19]. Of interest, we found increased subclinical atherosclerosis and immunoactivation in a study in vertically HIV-infected children and adolescents [20], and we have recently described in HIV-infected adults with long-term viral suppression that the CD4/CD8 ratio correlates with markers of age-associated disease [21]. Hence, if the CD4/CD8 ratio is further validated as a marker of the accelerated aging syndrome that characterizes treated HIV infection, HIV-infected children and adolescents failing to normalize the CD4/CD8 ratio despite ART should be carefully monitored for the prompt detection and early treatment of noninfectious disorders related to premature aging, as the effects of immunoactivation/immunosenescence on clinical outcomes will be probably stronger on vertically HIV-infected patients, whose immune system has developed in the presence of the virus since birth or even before. Also, the CD4/CD8 ratio might be of great interest for identifying the best candidates to be enrolled in clinical trials aiming to reduce immunoactivation. Importantly, as the CD4/CD8 ratio is available in clinical practice, its use to identify patients with higher levels of immunoactivation and consequently a higher risk of non-AIDS events might be easily implemented in clinical settings.

Our results demand a cautious interpretation given the small sample size and warrant confirmation in larger studies. Unfortunately, we were unable to explore the associations between the CD4/CD8 ratio, levels of bacterial translocation and the frequency of CMV-specific T-cells or CMV serostatus, which is a study limitation, as they could have helped explain the relationship between the CD4/CD8 ratio and immunosenescence in treated HIV infection.

In conclusion, our data suggest that a low CD4/CD8 ratio in vertically HIV-infected children and adolescents on ART is associated with increased levels of T-cells with markers of activation, senescence and activation/exhaustion, and a skewed T-cell phenotype from naïve toward effector memory. Thus, an inverted CD4/CD8 ratio may identify patients with ongoing immunoactivation despite ART, a finding that may have implications both in diagnostic and therapeutic settings.

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Conflicts of interest

There are no conflicts of interest.

Author's contributions to the work: T.S., study design, data analysis, and writing. S.S.-V., study design, data analysis, and writing. L.D., laboratory tests. M.G.-T., recruitment, data collection and analysis Hospital Doce de Octubre. MD: Gurbindo: recruitment, data collection and analysis Hospital Gregrorio Marañón. M.I.deJ.: recruitment, data collection and analysis Hospital La Paz. M.M., recruitment, data collection and analysis Hospital Carlos III. J.R., recruitment, data collection and analysis Hospital de Getafe. J.Z.: study design, statistical analysis, S.M.: study design, data analysis and writing. M.A. M.-F. study design, data analysis and writing.

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Summary: In a group of 37 vertically HIV-infected children, a low CD4/CD8 ratio was associated with increased levels of activated, senescent and activated/exhausted T-cells, and may be used as a marker of immunoactivation, both in diagnostic and therapeutic settings.

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Treatment-related changes in serum lipids and inflammation: clinical relevance remains unclear. Analyses from the Women's Interagency HIV study

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Among 127 HIV-infected women, the magnitude of high-density lipoprotein cholesterol (HDLc) increases after HAART initiation predicted the magnitude of concurrent decreases in inflammation biomarkers. After HAART initiation, changes in low-density lipoprotein cholesterol (LDLc) and inflammation were unrelated. In the same population, predicted risk of coronary heart disease, based upon levels of standard clinical risk factors, was similar before and after HAART. Thus, it remains unknown whether short-term treatment-related changes in standard risk factors may appreciably change risk of cardiovascular disease (CVD).

A recent article in *AIDS* by Piconi *et al.* [1] reported that among HIV-infected individuals, prothrombotic and inflammation factors were lower and metabolic factors (i.e. serum cholesterol and lipoproteins) were higher in persons on antiretroviral therapy (ART) than untreated persons. The authors concluded that HIV replication and inflammatory/thrombotic factors may be an important pathway to atherosclerosis in untreated HIV-infected individuals, whereas changes in metabolic factors may be important atherosclerosis risk factors in those using ART. Although an important contribution, the Piconi study lacked data on women, was limited by a cross-sectional study design and made no conclusions about changes after ART initiation in widely used clinical measures of future cardiovascular disease (CVD) risk.

We confirmed and extended the key conclusions of Piconi *et al.* [1] using longitudinal data from the Women's Interagency HIV Study (WIHS). Using data from a WIHS substudy of 127 HIV-infected women who initiated HAART while enrolled in the WIHS [2], we measured levels of lipids and inflammation factors at three semi-annual visits prior to first use of HAART and again at three semi-annual visits after first use of HAART. These data were used to examine the association between changes in serum lipids and concurrent changes in levels of inflammation-related biomarkers. Levels of high-density lipoprotein cholesterol (HDLc) increased after initiation of HAART (from 48 to 54 mg/dl), whereas low-density lipoprotein cholesterol (LDLc) increased only among the 67 women who initiated protease inhibitor-based HAART regimens (in PI-HAART users, 92–109 mg/dl, and in nonprotease inhibitor-based HAART users, 101–103 mg/dl). Regardless of the type of HAART regimen used, the magnitude of increase in HDLc was correlated with the magnitude of decrease in soluble CD14 (sCD14), tumor necrosis factor alpha (TNF-α), soluble interleukin 2 receptors (sIL-2R), interleukin 6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) (Fig. 1). Change in LDLc level was not associated with changes in inflammation biomarker levels, suggesting a largely inflammation-independent mechanism for increased LDLc with PI-HAART.

Among the same patient population, we also investigated the net effect of changes in lipid profile and other vascular risk factors after ART initiation on predicted risk of clinical CVD events. Hence, we calculated predicted coronary heart disease risk in HIV-infected women at visits shortly before initiating HAART, and again 18 months after beginning HAART. The Framingham risk score for estimating the 10-year risk of total coronary heart disease (including angina and fatal and nonfatal acute coronary events) [3] was calculated on the basis of age, total cholesterol (TC), HDLc, DBP and SBP,

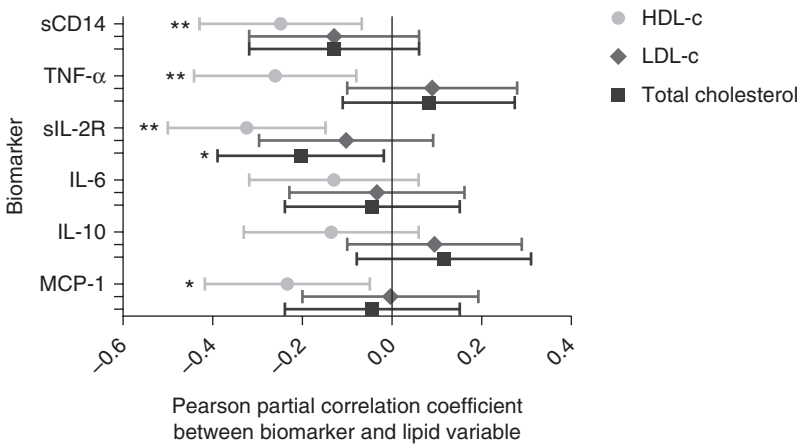

Fig. 1. Correlations of post-HAART changes in high-density lipoprotein cholesterol, low-density lipoprotein cholesterol and total cholesterol with post-HAART changes in inflammation-related biomarkers among HIV-infected women. Pearson partial correlations were computed between change variables; $^{*}P < 0.05$; $^{**}P < 0.01$; Pearson partial correlation coefficients are presented, with 95% confidence intervals; Adjusted for post-HAART change in $CD4^{+}$ T-cell count and post-HAART change in HIV RNA. HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol.

diabetes and current smoking status. We grouped women into low (10-year risk <15%), moderate (15–25%) or high risk (≥25%) categories, while placing all diabetic patients into the high-risk category [4,5]. We then determined the proportion of women who were reclassified after HAART initiation. Among all HIV-infected women prior to treatment, 84% had low predicted risk of coronary heart disease, 1% had moderate risk and 15% had high risk. After HAART initiation, 97% remained in the same risk category, 1% moved into a lower risk category and 2% moved into a higher risk category.

In summary, consistent with the findings from Piconi *et al.* [1], our data demonstrate the reciprocal relationship of inflammation and lipid perturbation in HIV-infected patients, while also suggesting that LDLc and inflammation are biologically discrete pathways that may alter atherosclerosis risk. We [2,6] and Piconi *et al.* [1], among others, have demonstrated that in HIV-infected patients, inflammation and lipid levels are associated with common carotid artery intima–media thickness, a measure of subclinical atherosclerosis. However, it can be difficult to translate findings from this subclinical atherosclerosis measure into clinically meaningful information on risk of CVD. At least among middle-aged HIV-infected women, we find little evidence that the net balance of short-term metabolic alterations related to HAART initiation would appreciably change future risk of CVD as measured by standard clinical risk factors. Therefore, as research into novel CVD biomarkers and long-term treated HIV natural history continues to mature, it will become increasingly important to evaluate the clinical relevance of changes in intermediate biomarkers.

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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CAPÍTULO 4

Disfunción de las HDL en relación a los estados de inflamación y activación inmune secundarios a la infección por el VIH.

JUSTIFICACIÓN Y OBJETIVOS

Numerosas publicaciones sugieren que las alteraciones metabólicas secundarias al TAR son una de las causas asociadas al incremento de enfermedades cardiovasculares descrito en la población infectada por el VIH. Las alteraciones lipídicas que con mayor frecuencia se asocian a los fármacos antirretrovirales son la hipertrigliceridemia y la disminución en los valores de HDL. De hecho, el descenso en HDL se ha asociado no solo al tratamiento, sino también a la propia infección. Las partículas de HDL presentan propiedades antiaterogénicas, pues son responsables del transporte reverso del colesterol, y además atenúan la inflamación y protegen de la oxidación a las lipoproteinas de baja densidad. Sin embargo, trabajos recientes sostienen que en determinadas circunstancias, estas propiedades pueden verse comprometidas, convirtiéndose estas partículas en proaterogénicas. Este cambio en las propiedades de las HDL se ha descrito en presencia de algunas infecciones virales. Sin embargo, y aunque algunos trabajos han estudiado la funcionalidad de las HDL en cuanto a transporte del colesterol en el contexto de la infección por el VIH, no hay datos a cerca de su función antiinflamatoria, que podría verse alterada en presencia de una situación de inflamación crónica y activación inmune como la que acompaña a la infección por el VIH. Determinar la existencia de una deficiencia funcional en las HDL secundaria a la infección por VIH podría contribuir a diseñar estrategias para la prevención y el tratamiento de la enfermedad cardiovascular.

Los objetivos de este capítulo son:

- Analizar *ex vivo* la función antiinflamatoria de las lipoproteinas de alta densidad en niños y adolescentes infectados por el VIH en relación a un grupo control, mediante ensayos de migración de monocitos.
- Determinar si existe asociación entre factores clínicos y virológicos y entre las diferentes familias de TAR y la funcionalidad de las HDL.
- Investigar si existe relación entre la PCR como marcador de inflamación y la función de las HDL en el contexto de la infección por el VIH.
- Investigar el posible papel del fenómeno de activación inmune en la funcionalidad de las HDL.

CONCLUSIONES

- En este estudio piloto se incluyeron un total de 14 adolescentes con infección por el VIH y seis adolescentes no infectados. Todos los pacientes se encontraban en TAR (7 recibiendo un régimen que incluía lopinavir/ritonavir, 4 atazanavir/ritonavir, y 3 efavirenz). Solo 9 pacientes (64%) presentaba CV indetectable (<50 copies/mm^3).
- No hubo diferencias entre casos y controles en cuanto a perfil lipídico, aunque los pacientes infectados por el VIH presentaban cifras de HDL ligeramente inferiores a los controles (52 mg/dL [43-60] vs 62 [55-73], *p*=0,090).
- Aunque los adolescentes infectados por el VIH presentaban un GIM ligeramente superior a los controles, la diferencia no fue estadísticamente significativa (0,43 mm ± 0,02 vs 0,42 ± 0,02, *p*=0,709).
- Los pacientes infectados por el VIH mostraron frecuencias superiores de linfocitos T CD4 activados (2,4 [1,7-3,4] vs 1,6 [1,5-1,8], *p*=0,052) y también CD8 (9,9 [3,1-20] vs 2,8 [1,7-3], *p*=0,018). Sin embargo, la diferencia en cuanto a PCR fue no significativa (0.08 mg/dL [0,02-0,28] vs 0,02 [0-0,04], *p*=0,193).
- La función de las HDL, definida por su capacidad para inhibir la migración de los monocitos, estaba disminuida en los pacientes infectados por el VIH, aunque la diferencia no alcanzó significación estadística (50% capacidad inhibitoria (±7) vs 58% (±9), p=0.083). En el grupo de pacientes con CV detectable la diferencia era mayor y estadísticamente significativa (48% (±8) vs 58% (±7), *p*=0,044).
- No se encontraron diferencias en cuanto a funcionalidad de las HDL entre las distintas combinaciones de antirretrovirales estudiadas.
- Se encontró una correlación positiva entre la disfunción de las HDL y el nadir de CD4 (*p*=0,043), pero no se encontró correlación con la CV (*p*=0,165). Las HDL más disfuncionales se encontraron en los sujetos con mayores niveles de activación en linfocitos T CD4 (*p*=0,018) y mayores cifras de PCR (*p*=0,009), y una tendencia hacia un mayor grosor íntima-media (*p*=0,071).
- Estos hallazgos sugieren que la disfunción de las HDL podría desempeñar un papel en el desarrollo de la enfermedad cardiovascular en el contexto de la infección por el

VIH. Si estos resultados se confirman, los pacientes infectados por el VIH podrían beneficiarse de estrategias orientadas a disminuir la inflamación y activación crónicas, así como a mejorar la calidad y no solo la cantidad de las HDL.

FUNCTIONALLY DEFECTIVE HIGH DENSITY LIPOPROTEINS (HDL) ARE RELATED TO HEIGHTENED T CELL ACTIVATION IN VERTICALLY HIV-INFECTED ADOLESCENTS

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ABSTRACT

We assessed high-density lipoprotein (HDL) anti-inflammatory properties in a cohort of vertically HIV-infected adolescents. We hypothesized that proatherogenic mechanisms related to inflammation and immune activation during HIV-infection may impair HDL functionality and impact on the atherosclerotic burden. Compared to healthy controls, HDL from HIV-infected adolescents presented impaired functionality, as determined by its ability to inhibit monocyte chemotaxis in vitro, which correlated with detectable viral loads (p=0.044), lower CD4 nadir (p=0.043), increased levels of CD4 T-cell activation (p=0.018), higher C-reactive protein (p=0.009) and a tendency towards thicker carotid Intima-media thickness (p=0.071).

Keywords: HDL dysfunction; HIV; vertical transmission; adolescents; immune activation; CRP; IMT.

FUNCTIONALLY DEFECTIVE HIGH DENSITY LIPOPROTEINS (HDL) ARE RELATED TO HEIGHTENED T CELL ACTIVATION IN VERTICALLY HIV-INFECTED ADOLESCENTS

Introduction

Lipid abnormalities partially related to antiretroviral treatment (ART), are thought to be one of the underlying causes of the increased atherosclerotic burden described in HIV-infected patients [1]. However, there is mounting evidence supporting the role that up-regulation of inflammatory pathways and increase of activated T-cells despite modern ART regimens have on the atherogenic process during HIV infection [2,3]. Persistent inflammation and immunoactivation during HIV-infection are thought to be strong predictors of disease progression, leading to immunosenescence, premature aging and adverse outcomes [4]. In fact, inflammatory markers, and particularly high-sensitivity C-reactive protein (CRP), have demonstrated to strongly predict cardiovascular events in population with and without HIV infection [5,6].

ART related dyslipidemia is characterized by hypertriglyceridemia and decreased high-density lipoproteins (HDL). This decrease in HDL has been described to be related not only to treatment, but to the infection itself [7,8]. HDL particles are known to possess key atheroprotective properties: they display elevated cellular cholesterol efflux capacity, protect LDL particles against oxidative stress and attenuate inflammation. However, under certain metabolic and inflammatory conditions the anti-atherogenic properties of HDL may be compromised [8–10]. Functionality of HDL in terms of reverse cholesterol transport has been described to be reduced during HIV infection [11] and, in general population, HDL efflux capacity has been recently shown to be a strong inverse predictor or coronary disease [12]. Although it is known that HDL loses its anti-inflammatory properties during acute influenza infection [10], to our knowledge no studies to date have addresses the effect of HIV on the anti-inflammatory properties of HDL.

Unraveling the underlying mechanisms of the atherogenic process during HIV infection is warranted in order to effectively prevent cardiovascular disease. We hypothesized that deficient HDL function and subnormal HDL levels may act synergistically to accelerate atherosclerosis during HIV disease. In this pilot study, we assessed HDL functionality ex vivo in a group of vertically HIV-infected adolescents, a population at high risk for early cardiovascular disease, in comparison to healthy controls. Simultaneously, we studied its association with clinical variables –including carotid intima-media thickness (IMT) – CRP and T-cell activation.

Patients and methods

Vertically HIV-infected and uninfected adolescents participating in a multicenter cardiovascular risk study were included in this substudy. Exclusion criteria included acute or opportunistic infections and chronic inflammatory diseases, as well as previous diagnoses of hyperlipidemia, hypertriglyceridemia or use of any medication other than ART. Medical records were carefully reviewed at interview and a thorough clinical examination was performed. Clinical and immunovirological data were extracted from the Cohort of Madrid collaborative Pediatric HIV Study database. The study was approved by the Ethics Committee and all participants and legal guardians gave written informed consent.

A sample of fasting venous blood was obtained. Plasma viral load (VL) was measured using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Inc, Branchburg, NJ), detection limit of 50 copies/mm^3. $CD4^+$ and $CD8^+$ T-cell counts were using a Gallios flow cytometer, and data analyzed using Kaluza software (Beckman Coulter®, California, USA). T-cell activation was characterized by $HLADR^+CD38^+$ coexpression determined by immunophenotyping from peripheral blood mononuclear cells (PBMCs) obtained from fresh blood. High sensitivity CRP was analyzed from frozen plasma samples with a commercial ELISA kit (eBioscience, Inc, San Diego, CA), with a detection threshold of 3 pg/μL.

HDL were isolated by sequential density ultracentrifugation from frozen samples and tested *in vitro* for its ability to inhibit the chemotaxis of monocytes (THP-1 cells), assessed by Transwell® cell culture chambers. A total of 100,000 cells resuspended in 100 µL RPMI 1640 containing 0.5% fetal calf serum (FCS), were added to the upper compartment of the insert with HDL isolated from every patient at a concentration of 100 µg/mL, and incubated at 37ºC for 30 minutes. After incubation, 6µg of recombinant human *monocyte chemo attractant protein* (MCP-1) diluted in 100 µL RPMI 1640+0.5% FCS was added to the lower chamber. THP-1 cells were allowed to migrate for 2h at 37ºC. Migrated cells in the lower chamber were then counted by flow cytometry. HDL functionality was defined as ability to inhibit monocyte chemotaxis, and expressed as percentage of inhibition of migrated cells, with respect to migration in the presence of MCP-1 alone. All experiments were run in duplicate.

IMT was examined using a CX50 ultrasonographic portable equipment, and specific IMT detection software QLab (Philips Medical Systems, Inc., Eindhoven, The Netherlands). Measurements were made bilaterally at the common carotid artery (1-2 cm proximal to the bulb), as previously described [13]. Images were read by an experienced cardiologist, blind to the HIV status.

Continuous variables were expressed as median and interquartile range (IQR), and categorical variables as absolute counts and percentages. Given the sample sizes and the non-normal distribution of some of the variables, non-parametric statistical tests were used. Comparisons between groups were analyzed using the Wilcoxon rank sum test. Linear regression and the Spearman's correlation coefficient were used to analyse the correlation between continuous variables. Independent associations of HDL functionality and a limited subset of independent clinical and immunovirological continuous variables were explored. The null hypothesis was rejected by a type I error <0.05. Statistical analyses were performed using Stata v. 12.0 (StataCorp LP College Station, Texas, USA).

Results

Study population and between group comparison of clinical characteristics

We included 14 vertically HIV-infected adolescents and 6 uninfected controls. All HIV-infected subjects were on ART (7 receiving a lopinavir/ritonavir containing regimen, 4 atazanavir/ritonavir, and 3 efavirenz), but only 9 (64%) were virologically suppressed. Median CD4 nadir was 371 cells/mm^3 [166-572], and median CD4 count 841 cells/mm^3 [556-1100]. Median VL for the unsuppressed group was 268 cop/mL [188-5839].

Mean age was 16.1 years ± 1.9 in the HIV-infected group and 17.3 ± 2.9 in the control group. There was a non-significant higher proportion of females in the HIV-infected group (71% vs 50%, p=0.369). Groups were similar in terms of BMI, blood pressure and anthropometric measurements. Of note, there were no significant differences regarding levels of total cholesterol (165 mg/dL [134-186] in the HIV-infected group vs 153 [144-214] in the uninfected subjects), LDL (90 mg/dL [77-105] vs 87 [59-111]) or triglycerides (116 mg/dL [75-143] vs 90 [59-150]) (all p>0.10). HDL levels were slightly decreased in HIV-infected subjects (52 mg/dL [43-60] vs 62 [55-73], p=0.090). Although median IMT was slightly higher in the group of HIV-infected patients, the difference between groups was non-significant (0.43 mm ± 0.02 vs 0.42 ± 0.02, p=0.709). HIV-infected patients displayed higher frequencies of activated CD4 T-cells (2.4 [1.7-3.4] vs 1.6 [1.5-1.8], p=0.052) and CD8 T-cells (9.9 [3.1-20] vs 2.8[1.7-3], p=0.018) but the difference in CRP did not reach significance (0.08 mg/dL [0.02-0.28] vs 0.02 [0-0.04], p=0.193).

HDL functionality and its relations to clinical variables, inflammation and T-cell activation.

HDL functionality was impaired in HIV-infected subjects compared to uninfected subjects, although the difference did not reach statistical significance (50% inhibition of chemotaxis (±7) vs 58% (±9), p=0.083). However, the subgroup of HIV-infected individuals with HIV RNA >50 cop/mL exhibited significantly impaired HDL functionality compared to healthy subjects (48% (±8) vs 58% (±7), p=0.044) (Figure 1A-B). No differences were detected within ART-groups (Figure 1C).

Figure 1. HDL functionality, HIV status and antiretroviral regimen.

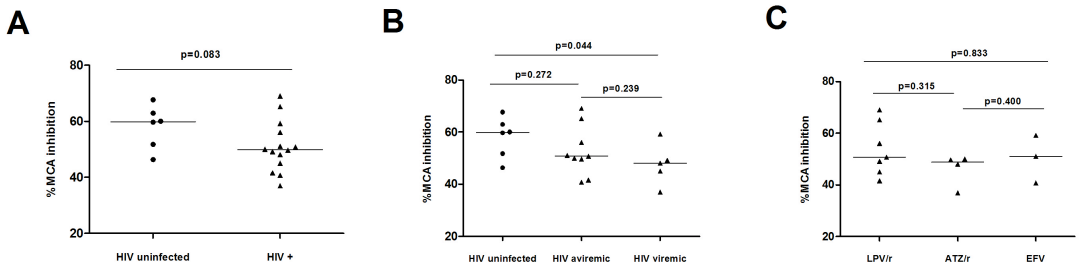

Figure legend: Circles correspond to uninfected subjects, triangles to HIV-infected individuals. Horizontal lines represent median values. HIV-infected subjects displayed non-significant lower HDL anti-inflammatory function, measured as percentage of inhibition of monocyte chemotaxis (MCA), compared to uninfected controls (Fig 1*A*). The difference reached statistical significance when only non-suppressed patients were included in the analysis (Fig 1*B*). No differences were found when ART regimens were compared (Fig 1*C*). *HDL; high density lipoprotein, MCA; monocyte chemotaxis assay, ART: antiretroviral treatment, LPV/r; lopinavir/ritonavir, ATZ/r; atazanavir/ritonavir, EFV; efavirenz.*

HDL functionality positively correlated with $CD4^+$ T-cell nadir (Rho: 0.55, p=0.043) (Figure 2A), but no association was found between the percentage of inhibition of monocyte migration and CD4 or CD8 counts (data not shown). Although unsuppressed patients showed lower HDL functionality, the negative correlation between HDL functionality and viral load did not reach significance (Rho: -0.39, p=0.165) (Figure 2B). Spearman's correlation coefficient was non-significant for CRP (Rho:-0.33, p=0.149) but the association was significant in a linear regression analysis (β:-26.4, p=0.009) (Figure 2C). HDL function tended to inversely correlate with IMT (Rho:-0.41, p= 0.071) (Figure 2D). Finally, HDL functionality inversely correlated with the frequency of $HLADR^+CD38^+CD4^+$ T-cells (Rho: -0.49, p=0.028), but not with the frequency of $HLADR^+CD38^+CD8^+$ T-cells (Rho:-0.28, p=0.095) (figure 2E – 2F).

Figure 2. Correlations between HDL functionality and clinical variables, CRP and T-cell activation.

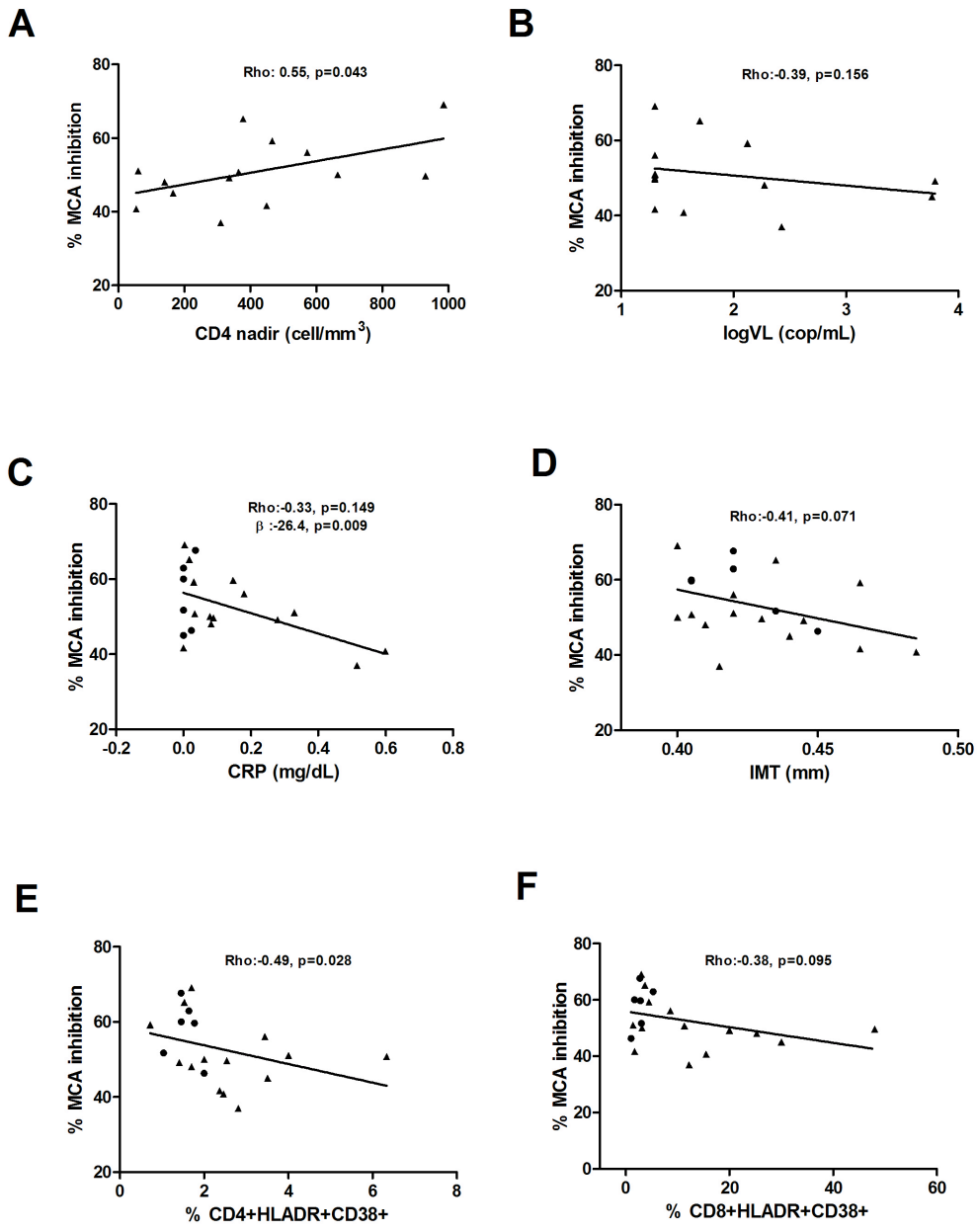

Figure legend: Correlations between HDL functionality, measured as the percentage of inhibition of monocyte migration, and different variables. There was a positive correlation between HDL functionality and CD4 Nadir (Fig 2*A*) and a non-significant negative correlation with viral load (Figure 2*B*). HDL anti-inflammatory ability tended to negatively correlate with C-reactive protein levels (Fig 2*C*) and IMT (Fig 2*D*). HDL functionality was negatively correlated with the percentage of activated $CD4^+$ T-cells (Fig 2*E*), but not with the frequency of activated $CD8^+$ T-cells (Fig 2*F*). *HDL; high density lipoprotein, MCA; monocyte chemotaxis assay, CRP; C-reactive protein, IMT; intima-media thickness, logVL; Viral load logarithm.*

Discussion

In this study, we found a decrease in HDL anti-inflammatory function in vertically HIV-infected adolescents, which was significant in patients with detectable HIV RNA compared to healthy controls. HDL functionality tended to inversely correlate with IMT, CRP and frequencies of T-cells expressing markers of activation. These findings provide new insight to previous studies describing relationships between cardiovascular disease and chronic inflammation/immunoactivation during HIV-infection, since they suggest that impairment of the anti-inflammatory properties of HDL particles might be contributing to atherosclerosis in young vertically-HIV infected individuals.

With the advent of combination therapies, vertically HIV-infected children are expected to live many years. However, the increased risk of non-AIDS related morbidity observed in adults –especially cardiovascular disease– is becoming a major concern both for clinicians and researchers, as it has been shown that atherosclerosis is already present in HIV-infected children [13,14]. Understanding the mechanistic pathways of cardiovascular disease in the very unique population of vertically-HIV infected patients is therefore warranted, in order to design strategies to prevent future cardiovascular events.

Although the underlying mechanisms of the atherogenic process are still unclear, therapeutic approaches in adults have mostly focused on decreasing low density lipoprotein (LDL) levels by using mainly statins. The use of statins has attracted the attention of clinicians treating HIV-infected patients, since statins have shown to reduce cardiovascular risk in the general population, and particularly among individuals with low levels of systemic inflammation, which is a hallmark of HIV infection. In fact, CRP levels after statin therapy have been demonstrated to predict cardiovascular events, independently of the decreased achieved in LDL [15]. Consistent with these findings, results from our study suggest that strategies aiming to decrease inflammation/inmmunoactivation might be useful in order to restore HDL atheroprotective properties, especially in the setting of HIV infection. If our findings are further confirmed, therapeutic approaches targeting not just the quantity but the

quality of lipoproteins might be needed in order to achieve a reduction in cardiovascular risk, and especially beneficial for the unique population of perinatally HIV-infected subjects, with a lifetime exposure to ART and the immune disorders related to the infection.

According to our findings, achieving viral suppression is critical in order to preserve HDL functionality, although the limited number of patients with detectable viral loads in our study prevented us from making any further analysis. In addition, the different antiretroviral regimens studied did not seem to have any particular effect on HDL anti-inflammatory function.

Our results demand, however, cautious interpretation given the small sample size and the borderline statistical significance, and warrant confirmation in larger studies. Unfortunately, the reduced sample size of this pilot study limited the statistical power to detect significant associations. The intra-assay variability inherent to the monocyte migration assays is another important limitation to be taken into account, and all the experiments were run in duplicate for this reason. However, we believe that these findings suggest that HDL anti-inflammatory function may be playing a role in the atherogenic process that merits further investigation, since these findings may have implications both in preventive and therapeutic settings.

In conclusion, our data suggest that dysfunction of HDL particles is present in HIV-infected adolescents, especially if viral suppression is not achieved. Despite ART, ongoing inflammation/immunoactivation may compromise HDL functionality, contributing to the increased atherosclerotic burden described in HIV-infected patients. Thus, new therapeutic approaches aiming to raise HDL particles with preserved anti-atherogenic activity might be a promising strategy to improve the quality of life of HIV-infected subjects, and especially benefit patients at higher risk of non-AIDS events, such as vertically infected individuals.

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DISCUSIÓN

Los resultados de esta Memoria confirman, en la cohorte de pacientes pediátricos infectados por el VIH más larga estudiada hasta la actualidad, que los cambios estructurales de la pared arterial correpondientes a las primeras fases del proceso arteriosclerótico aparecen de forma precoz en los niños infectados por el VIH por transmisión vertical, y que pueden detectarse mediante técnicas de imagen ecográficas no invasivas. Este hecho pone de manifiesto la necesidad de inicidir en las medidas preventivas y el diagnóstico precoz de los factores de RCV en esta población, para prevenir la aparición de enfermedad cardiovascular precoz. Los resultados sugieren que el propio VIH juega un papel importante en el desarrollo del proceso arteriosclerótico. Por otro lado, los resultados obtenidos demuestran que la técnica de medición del GIM es segura, sencilla y resulta bien tolerada por la población pediátrica. Estos hechos podrían tener implicaciones prácticas en cuanto a valoración del RCV en niños con factores de riesgo en un futuro próximo. El estudio de un subgrupo de niños infectados por el VIH con buena situación clínica y sin alteraciones metabólicas no reveló elevación de biomarcadores cardíacos ni inflamatorios, lo que sugiere que un estricto control de las anomalías metabólicas secundarias al TAR puede ser una medida eficaz de reducción del RCV en esta población. Por otro lado, se estudió en profundidad el papel de las HDL en el contexto de la infección por el VIH, y se observó cómo estas moléculas pueden ver afectadas sus propiedades antiinflamatorias en el contexto de la inflamación y activación inmunes secundarias al VIH, pudiendo contribuir al aumento de RCV en estos pacientes. Además, se ha demostrado que los fenómenos de activación y senescencia inmune, así como algunos signos de envejecimiento clínico prematuro se encuentran presentes desde edades tempranas de la vida en los niños infectados por el VIH, hallazgos con importantes implicaciones diagnósticas y terapéuticas. Por último, se propone la utilización del cociente CD4/CD8 como marcador para identificar a aquellos sujetos que pese al TAR presentan mayores niveles de activación y senecencia inmune y por tanto mayor riesgo de enfermedades asociadas al envejecimiento prematuro.

VIH Y ARTERIOSCLEROSIS SUBCLÍNICA

Aunque las manifestaciones clínicas de la arteriosclerosis no se presentan hasta la edad adulta, se sabe que el proceso arteriosclerótico comienza en edades tempranas de la

vida[6]. En esta Memoria, se utilizaron técnicas no invasivas de medición del grosor íntima-media carotídeo como marcador subrogado de RCV para evaluar una población de niños y adolescentes infectados por el VIH. Se trata probablemente del marcador mejor conocido, más utilizado y validado de evaluación del riesgo cardiovasvcular, que ha demostrado asociarse de manera independiente a la aparición de eventos cardiovasculares en adultos[137]. Estudios previos han demostrado la elevación del GIM en niños con conocidos factores de RCV, tales como obesidad, diabetes o síndrome metabólico[35,150,158]. Varios estudios han evaluado la presencia de arteriosclerosis subclínica en niños infectados por el VIH utilizando el índice íntima-medio carotídeo. La mayoría, pero no todos, han descrito la presencia de remodelamiento de la pared arterial precoz en estos pacientes, aunque las diferencias descritas varían enormemente entre los diferentes estudios (en un rango que va desde 0,02 mm hasta 0,15 mm según las series), y no existe consenso acerca de la importancia de los diferentes factores implicados en el proceso[7,8,32,33,155]. Los tamaños muestrales reducidos, la falta de consenso en cuanto a las características del grupo control ideal, o las divergencias en cuanto a las técnicas utilizadas probablemente son la causa de esta divergencia de resultados entre los diferentes estudios. En esta Memoria se presentan los resultados correspondientes a la serie más larga estudiada hasta el momento, y que corroboran la hipótesis de que los pacientes infectados por el VIH de forma perinatal presentan signos precoces de enfermedad vascular subclínica. Aunque la diferencia encontrada en cuanto a grosor de las capas íntima y media arterial es pequeña y su significado clínico incierto, la presencia de estos cambios mínimos en la pared arterial resulta preocupante, ya que refleja el inicio de un proceso en individuos aún muy jóvenes y con un largo periodo evolutivo por delante de convivencia con factores de RCV. En población adulta, mínimos engrosamientos de la pared arterial se asocian a incrementos anuales importantes en el riego de infarto agudo de miocardio[159], por lo que los hallazgos presentados en esta Memoria se deben considerar seriamente, e interpretar como señales de un proceso biológico que requiere de la puesta en marcha de medidas preventivas y de diagnóstico y tratamiento precoz. Por otro lado, existen datos en la literatura científica que sostienen la hipótesis de que dichos cambios de la pared arterial no son irreversibles[47,153,154]. Se está por tanto a tiempo de introducir las modificaciones pertinentes en cuanto a estilo de vida, factores dietéticos, ejercicio, etc. orientadas a disminuir los factores de RCV en esta población. Pese a la creciente preocupación de la

comunidad científica ante el aumento de fenómenos cardiovasculares en población infectada por el VIH, no se dispone en la actualidad de guía clínicas de prevención y tratamiento específicas. Ya en 2011, McComsey et al. en su revisión del tema hacían hincapié en la necesidad de implementar las medidas preventivas, en concreto en el colectivo de pacientes de transmisión vertical, que debido al TAR está alcanzando en la actualidad la edad adulta[160]. Desde 2007, la Academia Americana de Cardiología reconoce la infección por el VIH como un factor añadido de RCV a considerar a la hora de establecer los puntos de corte para indicar el inicio del tratamiento farmacológico de la hiperlipemia[161]. Sin embargo, desde entonces varios autores han puesto de manifiesto el hecho de que, pese a la elevada prevalencia de dislipidemia descrita en las diferentes cohortes de niños y adolescentes en tratamiento antiretroviral, muy pocos pacientes reciben tratamiento hipolipemiante[126,127]. Estos estudios subrayan la necesidad de diseñar estrategias específicas de prevención del RCV en esta población, así como de llevar a cabo ensayos clínicos de tratamiento. Aunque indudablemente son otras las prioridades en el día a día en la consulta en cuanto al manejo de estos pacientes, con complicaciones frecuentes relacionadas con su enfermedad y también con su particular situación sociodemográfica, deber tenerse presentes las posibles complicaciones de la infección a medio y largo plazo, y entre ellas el aumento de RCV. Las consultas de pediatría representan el lugar ideal para incidir en los hábitos dietéticos y el estilo de vida de los pacientes, en un momento en que éstos no están aún consolidados.

DISCUSIÓN DE FACTORES FISIOPATOLÓGICOS IMPLICADOS EN EL PROCESO ATEROSCLERÓTICO

Factores cardiovasculares clásicos, variables asociadas a la infección por el VIH y su tratamiento

Resulta probable que haya influido en la ausencia de guías específicas de manejo clínico del RCV el hecho de que, como se ha mencionado anteriormente, el peso de los diferentes factores de riesgo en la compleja ecuación de la enfermedad cardiovascular no se conoce con exactitud, pese a haber sido objeto de intensa investigación. Aislar los factores de

riesgo relacionados con la infección, tales como la presencia de CV detectable, el efecto de la inmunodepresión o de los diferentes tratamientos antirretrovirales, de los factores de riesgo tradicionales (como el tabaquismo, la hipertensión arterial o la hipercolesterolemia) ha sido uno de los problemas fundamentales en los estudios realizados en adultos. Los estudios en población pediátrica ofrecen la oportunidad de analizar el problema en ausencia de factores de riesgo tradicionales, pero con frecuencia, las poblaciones estudiadas presentaban importantes alteraciones metabólicas y especialmente dislipemias secundarias a los primeros fármacos antirretrovirales, que una vez más hacían difícil evaluar los efectos de cada una de las variables a estudio. En el Capítulo 1 de esta Memoria se presentan los resultados principales del Estudio CaroVIH, ya publicados, en el que la inclusión de un amplio número de pacientes y un número igual de controles de características similares ha posibilitado la realización de un análisis ajustado por los diferentes factores de riesgo. Teniendo en cuenta los posibles efectos de la edad, el sexo, el índice de masa corporal, el hábito tabáquico o la hipercolesterolemia, la infección por el VIH se ha confirmado como un factor independiente de RCV.

Estudios previos han estudiado ampliamente el efecto de los fármacos antirretrovirales, y especialmente de los IP, sobre el sistema cardivascular. Sin embargo los resultados no han sido concluyentes[21,22,142]. Algunos de los estudios en población pediátrica parecían sugerir que el tratamiento con IP podía incrementar por sí mismo el riesgo de enfermedad cardiovascular[7,8]. Responder a esta cuestión resulta fundamental, ya que la familia de los IP es una de las más empleadas en el tratamiento de la infección por el VIH en edad pediátrica. Con intención de analizar este problema, la exposición a estos fármacos se analizó en este estudio utilizando datos retrospectivos de seguimiento extraidos de la base de datos de la Cohorte de Madrid de seguimiento de niños y adolescentes infectados por el VIH, que ha recopilado de forma retrospectiva la historia de tratamiento de todos los pacientes en seguimiento en la Comunidad de Madrid. La exposición a fármacos se ha medido por tanto en forma de variable contínua, lo que dota de mayor relevancia a los resultados en comparación con los presentados en estudios previos, que analizaban la exposición a las distintas familias de fármacos en forma de variable dicotómica. El remodelamiento arterial es un proceso contínuo, que requiere de la presencia continuada de un factor de riesgo, y por tanto era importante precisar la duración de la exposición a fármacos. Analizándolo de esta

manera, no solo no se ha confirmado la hipótesis de que los IP constituyen un factor de RCV, sino que en el análisis multivariante la exposición a IP práticamente alcanzaba significación estadística como factor de protección. De hecho, restringiendo el análisis a los individuos no fumadores y con infección de transmisión vertical confirmada, la exposición a IP se traducía en una disminución del riesgo de presentar elevación del grosor íntima medio carotídeo del 16% anual. Aunque lógicamente no se puede descartar que los IP puedan ejercer un efecto directo sobre la pared vascular, como se ha sugerido en estudios previos, los resultados presentados en el capítulo 1 inducen a pensar que en cualquier caso el beneficio que producen los IP es superior, al menos en cuanto a desarrollo de enfermedad vascular subclínica. Probablemente, este beneficio se deba al menos en parte a que los IP son fármacos que han resultado altísimamente eficaces en el tratamiento de la infección por el VIH en niños, logrando una buena recuperación inmune y manteniendo el estado de supresión viral. Dos de las variables asociadas con la infección por el VIH que se han relacionado de manera constante en la literatura con el aumento de eventos cardiovasculares son el recuento total y el nadir de linfocitos T CD4[99,145,162,163]. Los resultados expuestos en el capítulo 1demuestran cómo el nadir de CD4 se asoció a un mayor grosor ínitima-medio carotideo, apoyando una vez más la hipótesis que sugiere que los fenómenos asociados a la disfunción del sistema imnune juegan un papel en el desarrollo de enfermedad cardiovascular asociado a la infeccción por el VIH.

Activación y senescencia inmune

En un intento de profundizar en esta hipótesis, en un subgrupo de pacientes se analizó la frecuencia de linfocitos T CD4 y CD8 con fenotipo activado (HLADR+38+) y senescente (CD28-57+). Aunque los niños y adolescentes infectados por el VIH presentaban frecuencias mayores de CD4 activados, y aquéllos con CV detectable frecuencias significativamente superiores de células T CD8 activadas y senescentes, ninguno de estos factores de asoció de manera significativa con la presencia de mayor GIM, como se muestra en el capítulo 1. El reducido tamaño muestral de este subanaálisis, en el que solo se incluyeron 38 pacientes y 11 controles, podría haber limitado de manera importante la capacidad para detectar asociación entre las variables a estudio, junto con el hecho de que la variable GIM presentaba una escasa

dispersión. Sin embargo, estos resultados son similares a los presentados por Kaplan et al. en 2011, que en su estudio en mujeres adultas infectadas por el VIH describen asociación entre los fenómenos de activación y senescencia inmune y la presencia de placa a nivel de la carótida, y sin embargo la asociación con el GIM no resultó estadísticamente significativa[30]. También podría ocurrir que los fenómenos de activación de células T no ejerzan en los niños el mismo efecto patológico que se ha descrito en población adulta. En este sentido, los resultados en la literatura científica en cuanto a si la activación inmune durante la infancia se asocia a peor evolución y progresión a enfermedad son controvertidos; inicialmente algunos trabajos apuntaban a una mayor presencia de linfocitos T CD8 activados en los pacientes progresores rápidos[164], mientras que más recientemente, otros investigadores no han encontrado asociación con la progresión de la enfermedad ni con la afectación neurológica[106,107]. Ningún estudio hasta la fecha ha analizado su implicación en el desarrollo de enfermedad aterogénica durante la infección por el VIH de transmisión vertical, y sí que hay numerosos datos en población adulta que sostienen que la presencia de los fenómenos de activación y senescencia del sistema inmune tiene un efecto deletéreo en los pacientes infectados por el VIH, por lo que consideramos que estos resultados deben ser considerados con extrema precaución. Además, si bien es cierto que los niños y adolescentes incluidos en este estudio presentaban frecuencias de linfocitos T CD4 activados significativamente superiores a las de la población control, también lo es que estos niveles distaban mucho de los descritos en población adulta. Este fenómeno podría deberse al hecho de que la coinfección por CMV, que como se ha mencionado anteriormente es un factor de activación y senescencia inmunológica conocido, podría no ser tan frecuente en niños como lo es en adultos infectados por el VIH. De hecho, estudios recientes en población adulta sugieren que el papel del CMV en el desarrollo de enfermedad vascular es capital durante la infección por el VIH[100,101,165]. Lamentablemente, no se llevaron a cabo las determinaciones pertinentes durante el estudio, ni se dispone de datos recientes para corroborar o descartar la presencia de CMV en la cohorte de pacientes incluidos en el estudio, por lo que esta hipótesis no puede ser confirmada. Probablemente serán necesarios estudios de mayor envergadura y con seguimiento prospectivo para dilucidar si existe una asociación real entre los fenómenos de activación y senescencia inmune secundarios a la infección por el VIH y el proceso aterosclerótico.

Traslocación bacteriana, inflamación y activación de la inmunidad innata

Ya es un hecho indiscutible que el fenómeno de la traslocación bacteriana en la infección por el VIH juega un papel importante en cuanto a la progresión de la enfermedad. El paso de productos bacterianos al torrente sanguíneo contribuye al mantenimiento de un estatus inflamatorio crónico. Tanto el sCD14 como el CD163, marcadores de activación monocitaria, se asocian a progresión a enfermedad y mortalidad[111,112]. También se ha observado que monocitos y macrófagos son protagonistas en el remodelamiento de la pared arterial, y grandes liberadores de mediadores inflamatorios, y cada vez son más los investigadores que sostienen que la disfunción de la inmunidad innata juega un papel importante en el mantenimiento de la inflamación crónica y en consecuencia en el proceso de envejecimiento prematuro y desarrollo de complicaciones no asociadas a SIDA.

En el estudio presentado en el capítulo 2, actualmente aceptado para su publicación, marcadores de inflamación (PCR, IL-6, mieloperoxidasa), función endotelial (activador tisular del plasminógeno), moléculas de adhesión vascular (sVCAM y P-selectina) y marcadores de activación monocitaria se determinaron en un subgrupo de pacientes en TAR. El subgrupo de pacientes incluido en el estudio tenía una buena situación inmunológica y no presentaba alteraciones metabólicas importantes. En estas circunstancias, a excepción de un aumento en los valores de sVCAM, no se detectó elevación significativa de los biomarcadores de las diferentes vías relacionadas con la enfermedad cardiovascular. Obviamente, el tamaño muestral reducido, junto con el hecho de que la mayor parte de los marcadores siguen distribuciones no normales podría haber limitado la capacidad para detectar diferencias, por lo que los resultados se deben intepretar con precaución. Sin embargo, la ausencia de diferencias entre los grupos podría deberse precisamente a las particulares características de este subgrupo de pacientes, y especialmente a la ausencia de alteraciones lipídicas y el buen control inmunológico. Estos resultados concuerdan con los trabajos publicados por Miller et al[9,72], que sugieren que en pacientes pediátricos, la elevación de marcadores de RCV se asocia fundamentalmente a la presencia de alteraciones lipídicas y/o CV detectable. Estos resultados son por tanto esperanzadores, pues sugieren que los mecanismos proaterogénicos pueden ser parcialmente revertidos, siempre que la terapia antirretroviral se instaure con éxito y se logre un estricto control de las alteraciones

metabólicas secundarias al TAR. En ausencia de guías de manejo específico, un óptimo control de las dislipemias y otras alteraciones metabólicas asociadas a los antirretrovirales debe ser una prioridad. En base a los resultados obtenidos con este panel de biomarcadores cardíacos e inflamatorios, ninguna de las moléculas estudiadas se asoció de forma independiente con el grosor íntima-medio carotídeo, por lo que parece limitada su utilidad en cuanto a estratificación del RCV en pediatría.

Curiosamente, en este contexto de enfermedad tratada y con buen control, se encontró un aumento significativo en los valores de sCD14 en los niños infectados por el VIH, no asociada a la presencia de CV detectable, pero sí a la presencia de valores más altos de PCR. Los pocos estudios publicados que han analizado este marcador en población pediátrica infectada por el VIH han descrito un aumento desde el nacimiento en los pacientes infectados por transmisión perinatal[123], así como su escasa normalización tras iniciar diferentes líneas de tratamiento e independientemente de la supresión viral y la recuperación inmunológica[124]. Puesto que el sCD14 es un marcador de activación monocitaria y puede ser considerado un marcador indirecto de translocación bacteriana, estos estudios concluyen que el fenómeno de la traslocación bacteriana continúa pese al TAR también en los pacientes de transmisión vertical, y en mayor medida en aquellos casos en los que el tratamiento se instaura de manera diferida, sugiriendo que un inicio precoz del TAR puede influir en el grado de afectación del MALT, y por tanto también en los niveles posteriores de translocación bacteriana. Como se muestra en el capítulo 2, además de asociarse de manera directa a la elevación de PCR (β= 0,27, p=0,041), el sCD14 se asoció débil pero significativamente con el nadir de CD4 (por cada aumento de 100 celulas, β= -0,052526, p=0,045), como se muestra en la **Figura 7**, inédita.

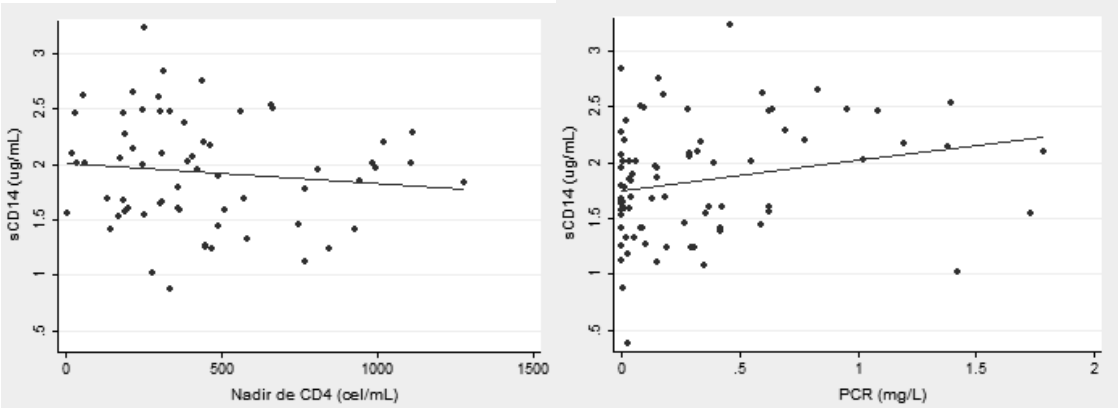

Figura 7. CD14 soluble en relación al nadir de CD4 (izquierda) y la PCR (derecha).

Aunque en no se encontró asociación directa entre los marcadores de activación monocitaria y la elevación de GIM, la presencia de valores altos de sCD14 en la población de niños y adolescentes infectados por el VIH y su relación con el nadir de CD4 apoya la hipótesis que sostiene que el grado de inmunodepresión alcanzado previo al TAR puede condicionar la afectación del MALT, y de manera importante la evolución posterior en cuanto a translocación bacteriana desde el intestino. Estos datos apoyan la hipótesis que defiende que es fundamental un inicio precoz del TAR, con el objetivo múltiple de evitar el estado de inmunodepresión y, sobre todo, disminuir la afectación del MALT y minimizar el reservóreo viral en el organismo[166]. Una vez más, estos hallazgos sugieren que probablemente los pacientes pediátricos sean los que más puedan beneficiarse de futuras estrategias orientadas a la disminución de la traslocación bacteriana, y por ende de la inflamación y la activación mantenida del sistema inmune.

Disfunción de las HDL

Mucho menos conocido resulta el papel que las las lipoproteinas de alta densidad pueden jugar en el reclutamiento y el paso de monocitos a través de la pared endotelial, y por tanto en el desarrollo de la placa de ateroma en el contexto de la infección por el VIH. Como se ha comentado anteriormente, algunas publicaciones sugieren que en determinadas circunstancias, y en concreto ante situaciones de inflamación y en presencia de algunas

infecciones, las HDL pueden ver comprometidas sus propiedades antiaterogénicas y convertirse en partículas proinflamatorias[130]. Este podría ser hipotéticamente el contexto de la infección por VIH. Como parte del estudio de factores potencialmente implicados en el desarrollo de arteriosclerosis durante la infección por el VIH, y para profundizar en el posible papel de las HDL disfuncionantes, se realizaron una serie de ensayos de migración de monocidos en presencia de MCP-1, orientados a testar *ex vivo* la capacidad antiinflamatoria de las HDL, y cuyos resultados se presentan en el capítulo 4, aún inédito. Las HDL de los adolescentes infectados por el VIH presentaban menor capacidad de inhibición de la migración monocitaria que las de los sujetos no infectados, aunque la diferencia solo alcanzó la significación estadística en el grupo de pacientes con CV detectable. Además, la disfunción de HDL se asoció a frecuencias más altas de células T activadas, y una tendencia a presentar valores más altos de PCR. No se encontró sin embargo efecto diferencial entre los diferentes regímenes de TAR estudiados. Estos hallazgos apoyan la hipótesis de que las HDL pueden ser disfuncionantes en el contexto de la inflamación crónica y la activación inmune secundaria a la infeccción por el VIH, y ponen de manifiesto una vez más la importancia de alcanzar y mantener la supresión viral en estos pacientes. De confirmarse, estos hechos podrían tener importantes repercusiones a medio y largo plazo, desde un punto de vista terapéutico. Por un lado, podría ser necesario en el contexto de la infección por el VIH la búsqueda de mecanismos orientados no tanto a aumentar la cantidad sino también la calidad/funcionalidad de las HDL. Y desde una perspectiva más amplia y como se comentaba al inicio de esta Memoria, cada vez más los fenómenos de inflamación y activación/senescencia inmunológica se van convirtiendo en una diana fundamental en la lucha por mejorar la calidad de vida y las expectativas de supervivencia de los pacientes. El desarrollo de estrategias orientadas a disminuir la inflamación en estos pacientes es hoy por hoy una necesidad, que podría tener efecto a nivel de múltiples órganos y sistemas, y entre otros el cardiovascular. En este sentido, los resultados de los ensayos clínicos recientes que se han realizado con estatinas apoyan esta hipótesis, puesto que demuestran una disminución en la incidencia de eventos cardiovasculares asociada sobre todo a una disminución en los marcadores de inflamación y no tanto a una mejora en los niveles de lípidos, tanto en población general[167,168] como en pacientes infectados por el VIH [169]. Estos resultados son aplicables incluso a personas sanas que ni siquiera han sido diagnosticadas de dislipemia[170],

lo que sugiere que, efectivamente, la inflamación juega un papel principal en el proceso aterosclerótico. Los pacientes de transmisión vertical pueden situarse entre los más beneficiados por estas nuevas estrategias, puesto que tienen por delante muchos años durante los cuales su organismo se verá sometido a los efectos deletereos de la inflamación, y los fenómenos de envejecimiento prematuro serán probablemente más patentes al tratarse de población más joven. En este sentido, resulta también fundamental desarrollar herramientas que permitan identificar a los sujetos en situación de riesgo, que serán por otro lado los que potencialmente más puedan beneficiarse de las nuevas terapias, y los candidatos ideales para participar en ensayos clínicos evaluando nuevas estrategias de tratamiento. Este objetivo se ha abordado en el capítulo 3 de esta Memoria.

EL COCIENTE CD4/CD8 COMO MARCADOR DE ACTIVACIÓN Y SENESCENCIA INMUNOLÓGICAS

En el contexto actual, en el que cada vez cobra mayor importancia en el manejo de los pacientes infectados por el VIH, el diagnóstico precoz y la prevención de las complicaciones asociadas al envejecimiento prematuro, numerosos grupos han orientado sus investigaciones hacia la identificación de biomarcadores accesibles a la práctica clínica que puedan ser de utilidad desde un punto de vista diagnóstico y terapéutico en la identificación de los sujetos en situación de mayor riesgo. A día de hoy múltiples moléculas siguen en estudio y, sin embargo, ninguna ha sido validada, por lo que el manejo de los pacientes sigue basándose en la optimización de dos variables; el recuento de CD4 y la CV.

Estudios realizados en población general han descrito lo que se ha dado en llamar "Perfil de riesgo Inmunológico" o *Immune Risk Profile*, definido por una serie de defectos del sistema inmune[171] asociados a morbi-mortalidad. Entre otras características, dicho perfil se distingue por la presencia de una acumulación de células $CD8^{+}CD28^{-}$ asociadas a la infección por CMV, y que se traduce en una inversión del cociente de CD4/CD8, que se ha asociado directamente con mortalidad por todas las causas[171–173]. Muchas de las alteraciones inmunológicas asociadas a este síndrome se han descrito también en el contexto de la infección por el VIH[174]; en ambos casos se describe expansión de células T específicas frente a CMV, descenso en el número de linfocitos naïve y aumento en las subpoblaciones de

memoria[175,176]. Antes de la introducción del TAR, la inversión del cociente CD4/CD8 era considerado un factor predictor de progresión a SIDA[177] y más recientemente se ha descrito su asociación con el desarrollo de linfoma Hodgkin y la prevalencia y volumen de placas coronarias[163,178], todas ellas patologías asociadas al proceso de envejecimiento prematuro. En base a esta evidencia, se decidió explorar su validez como marcador de activación y senescencia inmune.

En al capítulo 3 se resumen los hallazgos de este estudio ya publicado, realizado en en un subgrupo de niños y adolescentes con infección de transmisión vertical por el VIH y con una buena situación inmunológica, en el que se enconatró una fuerte correlación entre el cociente CD4/CD8 y el grado de activación, senescencia y agotamiento inmunológico. Pese a presentar buena recuperación inmunológica y un recuento medio de linfocitos T CD4 por encima de 500 cel/mm^3, más de un 40% de los pacientes presentaban inversión del cociente. La inversión del cociente se asoció además a la presencia de alteraciones en cuanto a las subpoblaciones linfocitarias, con cifras muy disminuidas de linfocitos naïve CD4 y CD8, y acumulación de subtipos celulares de memoria especialmente patente en el compartimento de células T CD8 (**Figura 8**, inédita).

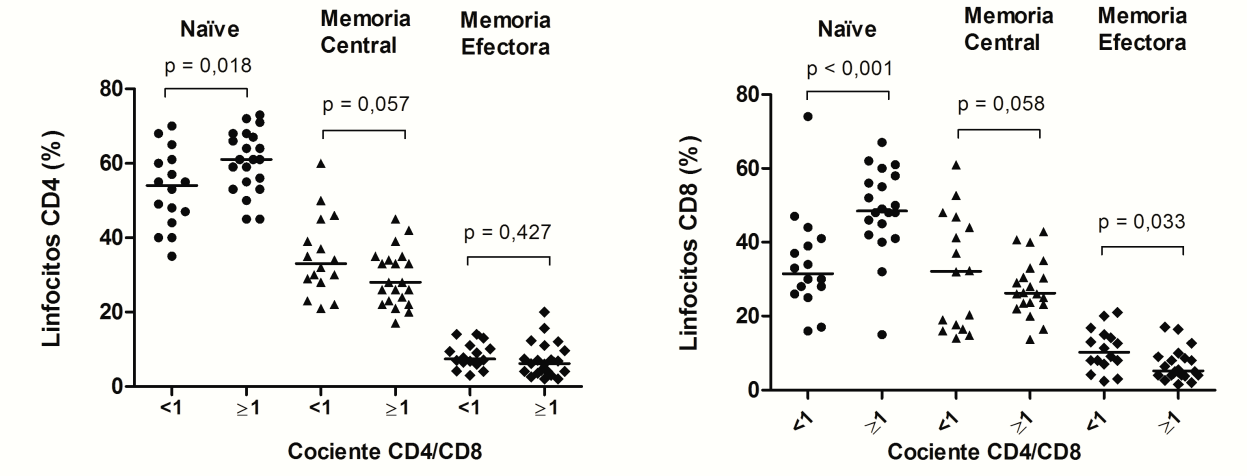

Figura 8. Subpoblaciones linfocitarias en función del cociente CD4/CD8.

El hallazgo de una inversión del cociente en un individuo con cifras normales de linfocitos T CD4 necesariamente implica la existencia de un recuento elevado de linfocitos T

CD8. Este hecho puede probablemente explicarse por la expansón clonal en estos pacientes de células T CD8, lo que por otro lado concuerda con el hallazgo en este grupo de una mayor diferenciación celular, de una mayor frecuencia de células con fenotipo de memoria y depleción en la población de linfocitos naïve. Lamentablemente, la contribución potencial de la coinfección por CMV a esta expansión clonal no se pudo analizar en este estudio. Su estudio arrojaría porbablemente luz sobre el fundamento etiopatogénico de la relación entre el cociente y el fenómeno de la inmunosenescencia. Pese al reducido tamaño muestral, que obliga a una interpretación cuidadosa de los resultados que se deberán confirmar en estudios de mayor envergadura, los hallazgos aquí presentados podrían tener importantes implicaciones desde un punto de vista diagnóstico y terapéutico. Si el cociente CD4/CD8 es definitivamente validado como marcador de inmunosenescencia, activación crónica y agotamiento inmunológico, los niños y adolescentes con inversión del cociente serían los candidatos ideales para participar en ensayos orientados a evaluar estrategias de control de la inflamación/activación en el contexto de la infección por el VIH. Por otro lado, la normalización del cociente podría convertirse en un objetivo clínico complementario a la hora de evaluar nuevos tratamientos para la infección. Y por último, la monitorización del cociente podría contribuir a mejorar el manejo de estos pacientes, permitiendo identificar a los sujetos con mayor activación pese al TAR, puesto que se trata de un marcador accesible a la práctica clínica habitual.

CONCLUSIONES

1. Los niños y adolescentes infectados por el VIH presentan un grosor íntima-medio carotideo elevado en comparación con controles sanos. Teniendo en cuenta el efecto de posibles variables de confusión, la infección por el VIH se asoció de forma independiente al incremento del GIM.
2. Las técnicas de evaluación ecográfica del GIM son seguras, sencillas, fácilmente aplicables en población pediátrica, y pueden ser útiles en la evaluación de pacientes en situación de RCV.
3. No se encontró asociación entre la exposición a tratamiento antirretroviral y el aumento del grosor íntima-medio carotideo. Por el contrario, un nadir de CD4 bajo se asoció de manera significativa a remodelamiento vascular, lo que sugiere que mecanismos inmunes están jugando un papel importante en el proceso aterosclerótico asociado a la infección por el VIH.
4. No se encontró aumento estadísticamente significativo en los valores de los biomarcadores cardiovasculares estudiados en los niños y adolescentes infectados por el VIH, a excepción de la molécula de adhesión sVCAM. Ninguno de los biomarcadores analizados se asoció al aumento del grosor íntima-medio carotideo, y ninguno ha demostrado utilidad en la evaluación del RCV asociado al VIH en niños y adolescentes.
5. Los niños y adolescentes infectados por el VIH presentaron elevación del marcador de activación monocitaria sCD14, pese a encontrase en tratamiento antirretroviral. El aumento en los valores de este marcador se correlacionaron positivamente con los valores de PCR, y negativamente con el nadir de CD4, pero no con un aumento del grosor íntima-medio carotideo.
6. Los pacientes infectados por el VIH presentaron niveles aumentados de linfocitos T CD4 activados. Sin embargo, dicha elevación no se asoció de forma significativa con aumento del GIM.
7. Los pacientes con CV detectable presentaron además mayor frecuencia de linfocitos T CD8 senescentes. Este aumento en el número de células T CD8 con fenotipo senescente tampoco se asoció a un aumento del GIM.
8. La presencia de un cociente CD4/CD8 invertido (<1) pese a una buena recuperación inmunológica en los niños y adolescentes infectados por el VIH en TAR se asoció

con un aumento en la frecuencia de linfocitos T CD8 activados, senescentes y con expresión de marcadores de agotamiento inmunológico, así como a disminución en la proporción de linfocitos naïve y aumento en el porcentaje de células de memoria efectora.

9. En los niños y adolescentes infectados por el VIH, la persistencia de un cociente CD4/CD8 invertido pese a una buena recuperación inmunológica con TAR podría ser un marcador de un estado subyacente de activación y senescencia inmune, útil en la identificación de sujetos con mayor riesgo de sufrir complicaciones asociadas al envejecimiento prematuro.
10. La capacidad antiinflamatoria de las lipoproteinas de alta densidad (HDL) era menor en el contexto de la infección por el VIH de transmisión vertical, especialmente en ausencia de supresión viral. Este hallazgo se asoció además con la presencia de mayor inflamación y activación inmune, y una tendencia a un aumento del GIM, lo que sugiere que la disfunción de las HDL está implicada en el proceso aterosclerótico asociado a la infección por el VIH.

CONCLUSIONS

1. HIV-infected children and adolescents displayed an increase in IMT when compared to uninfeced controls. After adjustment by potential confounders, HIV infection remained independently associated to increased IMT.
2. Ultrasound techniques for evaluation of IMT are safe and well tolerated and can be useful to evaluate cardiovascular risk in pediatric populations.
3. No association was found between exposure to ART and heightened IMT. However, a low CD4 nadir was significantly associated to a higher IMT, highlighting the role of immune disorders in the atherosclerotic burden associated to HIV disease.
4. No significant increase in the studied biomarkers was detected in HIV-infected children and adolescents, except for a significant increase in sVCAM. None of the studied biomarkers was related to thicker IMT. None of these markers demonstrated utility when evaluating cardiovascular risk in HIV infecetd children and adolescents.
5. HIV-infected children and adolescents, displayed significantly incresed levels of the monocyte activation marker sCD14, despite antirretroviral treatment. Elevation of sCD14 positively correlated with levels of CRP and negatively with CD4 nadir, but no association to IMT was found.
6. HIV-infected patients showed higher frecuencies of activated CD4 T cells. However, no association to increased IMT was detected.
7. HIV-infected individuals without viral suppression showed higher frecuencies of senescent $CD8^+$ T cells. Increase in the frequency of senescent $CD8^+$ T cells was unrelated to IMT.
8. HIV-infected children and adolescents displaying an inversion on the CD4/CD8 ratio (<1) despite good immune recovery under antiretroviral treatment showed higher frecuencies of activated, senescent, and exhausted CD8 T cells, and a skewed T-cell phenotype from naïve toward effector memory.
9. The CD4/CD8 ratio may be a useful marker to identify subjects with higher immunoactivation /immunosenescence despite ART, and thus at higher risk of premature ageing.
10. Dysfunction of HDL particles is present in HIV-infected adolescents, especially if viral suppression is not achieved, and related to increased levels of CRP, activated CD4 T cells and a tendency towards higher IMT. Ongoing

inflammation/immunoactivation despite ART may compromise HDL functionality, contributing to the increased atherosclerotic burden described in HIV-infected patients.

ANEXO I:
OTRAS PUBLICACIONES

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CARDIAC FUNCTION IN VERTICALLY HIV-INFECTED CHILDREN AND ADOLESCENTS IN THE ERA OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

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ABSTRACT

Background. Previous studies have evidenced the presence of premature atherosclerosis and ventricular dysfunction in HIV-infected adults. However, few studies have addressed this problem in vertically HIV-infected children and adolescents, and the long-term cardiac health of this unique population in the ART era is still unknown.

Methods. Ventricular function was evaluated cross-sectionally in a group of HIV-infected children and adolescents and healthy controls, using conventional echocardiography along with tissue Doppler imaging and strain analysis by speckle tracking. Simultaneously, measurements of carotid intima media thickness were performed.

Results. A total of 64 cases and 58 controls were included, mean age was 13.6 ± 5.4, 64% were females. All but 2 patients were on ART and 64% had undetectable viral load. HIV-infected patients showed higher intima media thickness (0.425±0.019 vs. 0.415±0.019, p=0.003). Statistically significant but clinically irrelevant differences were found among groups in ejection fraction and fractional shortening (66.1% and 36.2% in the HIV-infected group vs. 71.5% and 40.8% in the control group, respectively, p=0.001). There were no significant differences in diastolic function, tissue Doppler imaging or cardiac strain (longitudinal and rotational) between both groups. No associations were identified between echocardiographic parameters and current CD4 counts, CD4 nadir, HIV viral load, duration of antiretroviral therapy or ART regimens.

Conclusions. In a context of highly effective antiretroviral treatment, no evidence of cardiac abnormalities was detected using conventional and advanced ultrasound imaging techniques in this cohort of vertically HIV-infected children and adolescents, when compared to healthy controls.

Key words: HIV; children and adolescents; cardiac function; echocardiography; speckle tracking; IMT

CARDIAC FUNCTION IN VERTICALLY HIV-INFECTED CHILDREN AND ADOLESCENTS IN THE ERA OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

Introduction

Early diagnosed and treated vertically HIV-infected children can now expect to live for many years, as HIV infection has turned into a chronic condition. However, they are known to be a population at high risk for complications associated to a lifelong exposure to antiretroviral treatment (ART), as well as to the effects of the virus itself, and the infection-related inflammation and immune activation [1]. Among other ageing associated diseases, HIV-infected adults are known to have an increased cardiovascular risk, and the prevalence of cardiovascular disease is likely to rise as patients continue to age [2]. There is evidence that the atherogenic process described in HIV-infected adults starts early in childhood, and that vertically infected adolescents already present increased intima-media thickness [3–5]. Although its relation to premature atherosclerosis remains uncertain, ventricular dysfunction has also been described both in adult and pediatric HIV-infected populations [6–8].

Most of the evidence regarding the presence of cardiac abnormalities in vertically HIV-infected children comes out from the pre-ART era [7]. Echocardiographic abnormalities were common, persistent, and often progressive among untreated HIV-infected children, and included dilated cardiomyopathy with depressed LV contractility and LV dilation, heart failure, and aortic dilation. These findings were associated to an overall higher risk of all-cause mortality [7,9,10]. Very recently, results from a large, cross-sectional analysis comparing cardiac status of HIV-infected children in the pre and post-ART era and uninfected controls have been published [10]. Apparently, symptomatic cardiac disease associated with HIV infection has shifted with the highly effective antiretroviral treatment towards a mild and mostly asymptomatic condition, but it is still unclear whether this effect is the result of better immunological health, absence of co-infections, direct intramyocardial antiviral effect, or other mechanisms such as decrease in levels of systemic inflammation.

Over the past decades, novel and increasingly automated techniques for sophisticated analysis of cardiac mechanics have emerged. Among them, speckle tracking on the basis of displacement measurements has been suggested to be a more sensitive, load-independent measure to evaluate myocardial deformation or strain, able to detect cardiac dysfunction at an early stage. A recent report by Sims at al. supports the use of these new technologies in order to achieve an earlier detection of cardiac dysfunction in the specific population of HIV-infected children [11].

In this study, we aimed to perform a thorough evaluation of cardiac status using conventional echocardiography along with tissue Doppler imaging and speckle tracking, in a cohort of vertically HIV-infected children and adolescents and a group of uninfected controls. We simultaneously evaluated cardiovascular risk by means of Intima-media thickness (IMT), and analyzed the impact that clinical and immunovirological variables as well as exposure to ART may have on cardiac function.

Methods.

Study design and eligibility criteria. We performed a cross-sectional analysis from an ongoing prospective, longitudinal, multicenter observational study evaluating cardiovascular risk in a cohort of HIV-infected children and healthy uninfected controls. Between June and December 2011, children and adolescents attending the clinics of the six participating hospitals were prospectively enrolled. Exclusion criteria included acute or opportunistic infections, chronic inflammatory diseases, diabetes, kidney disease, hypertension, and family history of premature cardiovascular disease. Healthy adolescents from a high school in the same urban area and children attending the clinics for a routine cardiac examination were prospectively enrolled as controls. Additionally, a group of uninfected children born to HIV-infected mothers was included. Additional exclusion criteria for healthy controls included current infectious or inflammatory illnesses, chronic conditions and current use of medications. Controls were included with the goals of achieving a group with similar age, sex, ethnicity and body mass index (BMI) (±1 kg/m^2).

The study was reviewed and approved by the Ethics Committee and Clinical Research of the six participating Hospitals. All participants, parents or legal guardians and children over twelve years, gave written informed consent to take part in the study.

Clinical assessments

All participants underwent physical examination, including anthropometric and blood pressure measurements according to recommendations of the American Heart Association (AHA) [12], which were performed by the same trained technician. Hypertension was defined according to the p95 centile, adjusted by age and height [13]. Data regarding ART history and immunovirological variables were collected from the Cohort of Madrid collaborative Pediatric HIV Study database. To assess the effect of treatment, years of antiretroviral exposure were analyzed as a continuous variable.

Echocardiographic measurements

Resting cardiac echocardiography was performed by a single certified sonographer, blind to the HIV status, using a CX50 portable equipment equipped with an S5 multifrequency probe (Philips Medical Systems®, Inc., Eindhoven, The Netherlands), in the supine position.

Classic measures of left ventricle (LV) systolic function such as ejection fraction (EF) and fractional shortening (FS), as well as indicators of diastolic function such as mitral inflow E/A ratio and tissue Doppler-derived E/E´ratio, were evaluated. Parameters acquired from M-mode echocardiography included LV end-diastolic and end-systolic diameters, as well as septal and posterior wall thicknesses. Tissue Doppler velocities were assessed as the mean of three successive cardiac cycles. All measurements were performed according to the American Society of Echocardiography recommendations [14], and subsequently normalized by z score when needed [15].

Quantification of myocardial deformation (strain and torsion) by two-dimensional Speckle Tracking echocardiography (STE) was performed following consensus by the American and European Associations of Echocardiography (ASE/EAE) [16]. LV deformation parameters were analyzed offline by a trained cardiologist who was blind

to clinical status, using two-dimensional speckle-tracking echo software (QLAB Advanced Quantification Software version 7.1; Philips Medical Systems, Eindhoven, the Netherlands). Definition of these parameters has been described elsewhere [16]. Since STE relies on good image quality and high temporal resolution, all images that did not meet these criteria were excluded from strain specific analysis.

Strain is the percentage of myocardial deformation, that is, the fractional change in the length of a myocardial segment. This percentage is measured by continuously tracking natural acoustic markers (speckles) frame by frame (simultaneously in multiple areas within an image plane), providing local displacement information. Strain can have positive or negative values, which reflect lengthening or shortening, respectively. In our study, longitudinal strain (%) was computed from apical views (4-, 3-, and 2-chambers views).

The term LV rotation refers to myocardial rotation around the long axis of the left ventricle and is expressed in degrees. Generally, the base and apex of the ventricle rotate in opposite directions with counter clock-wise rotation of the apex and clock-wise rotation of the base around the LV long axis, when observed from the apical perspective. LV twist was assessed in our study from basal and apical short-axis views using the same frame rate, and was defined as the net difference between apical rotation and basal rotation at the maximal deformation point following current recommendations (Figure 1).

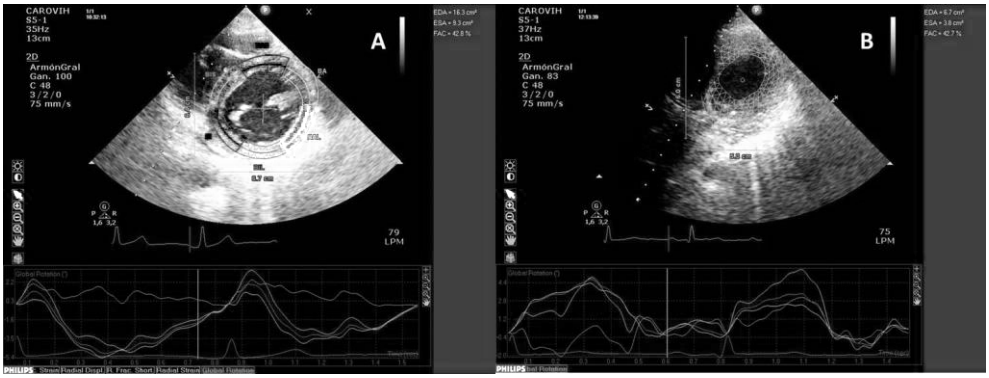

Figure 1. Assessment of left ventricle rotation by two-dimensional speckle tracking. LV rotation time curves (Bottom, both Panels) derived from two-dimensional speckle tracking analysis of the short-axis views at the base (Top, Panel A) and the apex (Top, Panel B) of the left ventricle.

Carotid artery ultrasound

IMT was examined simultaneously using the same CX50 portable equipment and specific IMT detection software –Qlab- (Philips Medical Systems, Inc., Eindhoven, The Netherlands). Measurements were made bilaterally at the common carotid artery (1-2 cm proximal to the bulb), as previously described [5] and digitalized. Mean value of the right and left measurement was then calculated and used for the analysis. All carotid ultrasounds were performed by a trained technician and read by an experienced cardiologist, blind to the HIV status.

Laboratory assays

Fasting blood samples were drawn for real-time measurements of insulin, glucose levels and lipid profile (total cholesterol, high-density lipoprotein cholesterol (HDLc), low-density lipoprotein cholesterol (LDLc), that were determined in the different participating hospitals using standard enzymatic methods. In the HIV infected group, plasma HIV-1 viral load (VL) was quantified using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Inc, Branchburg, NJ) with a detection limit of 50 copies/mm^3. Absolute and percentage CD4 counts were measured with standard flow cytometric methods.

Data analyses

Normally distributed quantitative variables were described as mean and standard deviation (SD), whereas continuous non-normally distributed variables were reported as median and interquartile ranges (IQR). Weight, height, BMI and hypertension were adjusted using *z* score according to the age and gender [13, 17], as well as ventricular dimensional parameters [15]. Means for variables with a normal distribution were compared using the Student's t-test. Non-parametric variables were examined using the Mann-Whitney and Kruskal-Wallis tests. Simultaneous independent associations between parameters of cardiac function and HIV-related variables were evaluated by logistic regression analysis. Statistical analyses were performed using Stata v. 12.0 (StataCorp LP College Station, Texas, USA).

Results

Study population and between group comparison of clinical characteristics

A total of 64 HIV-infected children and adolescents and 58 healthy volunteers underwent complete echocardiography including tissue Doppler imaging (TDI) and left ventricle rotational analysis. Main characteristics of both cohorts are summarized in table 1. Globally, mean age was 13.6 ± 5.4 (range 2.5 – 22.7). Most subjects were female (64%) and of Caucasian origin (74%). Most of HIV-infected patients (95.7%) had acquired HIV from mother-to-child transmission and all except two were receiving ART at the time of study evaluation. However, 23 (36%) of patients had detectable HIV RNA. Median viral load (VL) for this group was 1700 cop/mL [223-27600], and median logVL 3.23 cop/mL [2.34-4.44]. HIV-related parameters are described in table 1.

The control group was formed mainly by volunteers recruited from a high-school in the same urban area (n=25, 47%), children attending the outpatient clinics for a control echocardiographic study (n=17, 32%), and uninfected children born to HIV-infected mothers (n=12, 21%). Primary indications for the echocardiographic study were murmur (n=10), syncope (n=4), chest pain (n=2), and palpitations (n=1).

HIV-infected and uninfected subjects showed similar age, gender, and blood pressure. The frequency of hypertension was slightly higher in the control group, but the difference was non-significant. Although both groups had similar BMI, z score adjusted BMI was also higher in the control group. On the contrary, waist to hip ratio was lower in this group. No differences between groups were found regarding glycemia or lipid profile, except for total cholesterol/HDLc ratio and triglycerides. HIV-infected patients showed higher intima media thickness, consistently with previous findings in this cohort [5]. These observations are also summarized in table 1. None of the subjects was taking hypoglycemic agents, antihypertensive, statins or fibrates.

Table 1. Main characteristics of both cohorts

	HIV-infected subjects (N=64)	Control subjects (N=58)	p
Demographic data			
Age (years)	14 (5.3)	13.3 (5.4)	0.639
Female, N (%)	42 (65.6)	36 (62)	0.683
Caucasian, N (%)	46 (72)	44 (76)	0.622
Clinical variables			
Heart rate (beats/min)	74 (15)	79(13)	0.069
Systolic blood pressure (mmHg)	108 (12)	113 (15)	0.148
Diastolic blood pressure (mmHg)	62.7 (8.6)	65.3 (10.2)	0.370
Hypertension, N (%)	1 (3.2)	4 (11.1)	0.118
Smokers, N (%)	9 (14)	6(16)	0.726
BMI (kg/m²)	19.3 (3.8)	20.3 (3.3)	0.276
z score BMI*	0.00 (-1-0.5)	0.00 (-0.5-1)	0.018
Waist (cm)	67.3 (11)	67.9 (10.9)	0.775
Hip (cm)	76.1 (13.7)	84.6 (15)	0.001
Waist-to-hip ratio	0.88 (0.6)	0.85 (0.8)	<0.001
Laboratory measurements			
Glycemia (mg/dL)	88.2 (8.6)	85.8 (9.6)	0.586
Total cholesterol (mg/dL)	171.9 (40)	162.8 (36.1)	0.456
HDLc (mg/dL)	49.8 (11.3)	57.1 (16.4)	0.254
LDLc (mg/dL)	97.5 (36)	90.9 (32.3)	0.573
Total cholesterol/HDLc	3.6 (1)	2.9 (0.7)	0.013
Triglycerides (mg/dL)*	98 (72-146)	55 (48.5-73.5)	0.015
Intima-media thickness (mm)	0.425 (0.019)	0.415 (0.019)	0.003
HIV-related parameters			
On ART, N (%)	62 (96.9)		
Vertical transmission, N (%)	61 (95.7)		
HVC coinfection, N	1		
Viral Load <50 cop/mL , N (%)	41 (64)		
CD4 count (cell/mL)*	727 (535- 1012)		
Nadir CD4 (cell/mL)*	341 (198.5 - 604.5)		
Cumulative exposure to PI (years) *	6.6 (2.4-12.1)		
Cumulative exposure to NNRTI (years)*	1.4 (0.0-6.9)		
Cumulative exposure to NRTI (years)*	10.6 (6.6-14.2)		

Results are expressed as mean (standard deviation), except otherwise specified.
**Expressed as median (IQR)*
Hypertension: Systolic blood pressure >p95 of reference values
Abbreviations; BMI, body mass index [calculated as weight in kilograms divided by the square of height in meters; HDLc, high-density lipoprotein cholesterol; LDLc, low-density lipoprotein cholesterol; IMT, carotid intima media thickness; ART: antiretroviral treatment; PI, protease inhibitor; NNRT, non-nucleoside analogue reverse-transcriptase inhibitors; NRTI, nucleoside analogue reverse-transcriptase inhibitors; VL, viral load

A total of 33 HIV-infected subjects and 35 uninfected controls were included in the longitudinal strain analysis. Main characteristics of the subjects included in this subanalysis were very similar to the study population, with a mean age of 14.1 ± 5 years in the HIV-infected vs. 13 ± 5.6 in the control group, and a proportion of females of 66% vs. 60% (both comparisons, p>0.05.).

Echocardiographic findings

Baseline echocardiographic parameters are summarized by HIV status in Table 2. Both ejection fraction and fractional shortening –a measure of left-ventricular systolic performance– were slightly lower in the HIV-infected group compared to the control group (66.1 vs. 71.5%, p=0.001; 36.2 vs.40.2%, p<0.001, respectively). Only 4 (6%) patients had evidence of LV systolic dysfunction (defined as an LVEF and LVFS less than 55% and 27%, respectively) versus none of the controls (p>0.05). LV end-systolic diameter, but not end-diastolic diameter, was increased in the HIV-infected patients, and the difference remained significant when adjusted by z score (all p<0.05). Other parameters which reflect systolic performance of the right ventricle [tricuspid annular plane systolic excursion (TAPSE), and tissue Doppler-derived tricuspid annular systolic peak velocity] or the left ventricle [tissue Doppler-derived mitral annular systolic peak velocity] did not differ between groups.

There were also no differences between groups regarding septal or posterior wall thickness. When compared to reference population-based values, all parameters – including LV end-systolic diameter – were within the normal range both for the infected and uninfected individuals [15]. No differences between groups were found regarding conventional diastolic function parameters as E/A ratio on pulse wave Doppler assessment of mitral valve inflow or advanced diastolic function evaluation parameters obtained by tissue Doppler imaging, such as the E/E´ratio.

Speckle tracking analyses, including longitudinal LV strain and LV rotational motion, showed no differences between groups. Parameters of cardiac strain analysis by speckle tracking are also shown in Table 2.

Table 2. Echocardiographic parameters

	HIV-infected subjects (N=64)	Control subjects (N=58)	p
Conventional echocardiographic parameters			
LV end-diastolic diameter, mm	39.97 (5.9)	40.06 (6.1)	0.902
Z score adjusted*	-0.39 [-0.85,-0.16]	-0.55 [-1.06, 0.1]	0.282
LV end-systolic diameter, mm	25.5 (4.6)	23.7 (4.5)	0.027
Z score adjusted*	-0.2[-0.78,0.2]	-0.7[-1.0,-0.28]	0.001
Septal wall thickness, mm	9.3 (2.3)	9.2 (2.3)	0.708
Z score adjusted*	0.68 [0.06, 1.06]	0.34 [-0.13, 1.04]	0.409
LV posterior wall thickness, mm	7.6 (1.89)	7.9 (1.9)	0.491
Z score adjusted*	-0.34 [-0.97, 0.37]	0 [-0.8, 0.72]	0.336
LVFS, %	36.2 (6.4)	40.8 (6.6)	<0.001
LVEF, %	66.1 (8.4)	71.5 (7.5)	0.001
MA TDI S´ velocity, m/s	10.5 (4.7)	10.8 (2.2)	0.657
E/A ratio	1.78 (0.42)	1.83 (0.48)	0.244
E/E´ratio	5.26 (1.1)	5.07 (1.0)	0.400
TA TDI S´ velocity, m/s	13.0 (2.1)	13.3 (2.5)	0.430
TAPSE, mm	21.7 (4.4)	22.9 (5.7)	0.194
Speckle tracking analysis			
Longitudinal LV strain, %	-21 (3.4)	-20.3 (4.3)	0.867
LV rotation, degrees	6.1 (2.3)	5.5 (2.0)	0.091

Data are expressed as mean (SD), except otherwise specified.

* Expressed as median [IQR].

Abbreviations: *LV; left ventricle; LVFS, left ventricle fractional shortening; LVEF, left ventricle ejection fraction; MA TDI S´ velocity, mitral annular tissue Doppler-derived systolic wave velocity; E/A ratio, ratio between E and A waves of mitral valve inflow; E/E' ratio, ratio between E wave of mitral valve inflow and tissue Doppler-derived E´ wave; TA TDI S´ velocity, tricuspid annular tissue Doppler-derived systolic wave velocity; TAPSE, tricuspid annular plane systolic excursion.*

In order to adjust for the potentially deleterious effect of intra-utero exposure to ART on cardiac status, a sub-analysis was performed restricting the control group to unexposed individuals. Again, no differences were found between the 64 HIV-infected patients and the 46 controls, neither by conventional nor by speckle tracking analysis (data not shown).

HIV serostatus, HIV-related variables and echocardiographic parameters

Consecutive multivariate models were built to explore the associations between the different echocardiographic parameters and HIV status, controlling by potential confounders. After adjustment by age, gender and BMI, no association was established between HIV seropositivity and any of the parameters studied, except for a slightly

lower ejection fraction and fractional shortening [EF, β: -5.80, CI: -9.34, -2.25, p= 0.002 and SF, β: -5.04, CI: -7.99,-2.09, p=0.001] and increased Z score-adjusted left ventricle end-systolic diameter (β: 2.01, CI: 0.43,3.6, p=0.014).

Neither the adjusted nor the unadjusted analysis detected associations between any of the evaluated echocardiographic parameters and current CD4 T-cell counts or CD4 nadir. There was no evidence for a relationship between cardiac function or structure and current plasma HIV RNA level, duration of either protease inhibitor or non-nucleoside analogue use (all p>0.10). There was no correlation between any of the parameters evaluating cardiac function and intima media thickness.

Discussion

In this cross-sectional study, echocardiographic parameters were analyzed in a cohort of ART-treated HIV-infected children, and compared to a group of healthy controls. Both groups were similar in age, gender, ethnicity, BMI and proportion of smokers, as all of these factors are known to affect cardiovascular risk in the general population. Not unexpectedly, there were differences between groups regarding anthropometric measurements and lipid profile, although only differences in total cholesterol/HDLc ratio and triglycerides reached statistical significance. Despite having thicker IMT, no clinically relevant differences were found between HIV-infected and uninfected individuals regarding systolic or diastolic function of the ventricle. Similarly, the use of novel and more sensitive echocardiographic techniques, i.e., speckle tracking, revealed no differences.

Our findings are consistent with the recently published results from a cross-sectional analysis of the Adolescent Master Protocol of the Multicenter Pediatric HIV/AIDS Cohort Study, which strongly suggest that ART has a cardio protective effect, and that cardiac abnormalities are mostly mild and asymptomatic in the post-ART era [6]. According to this study, although overall ART has a cardio protective effect, treated vertically HIV-infected adolescents still present clinically non-significant lower mean LV fractional shortening z scores, and higher LV end-systolic dimension mean z scores,

compared to exposed HIV-uninfected controls [10]. The etiology of myocardial involvement in HIV infection is complex and not completely understood. Data from the preART era demonstrate that untreated HIV-infected patients show higher rates of pulmonary hypertension and cardiac abnormalities, suggesting a possible effect of the virus itself on the myocardial cells [9,18]. In fact, the study of uninfected children born to HIV-infected mothers indicates that the intrauterine exposure to the virus might be playing an important role in cardiac status [10]. Nucleoside reverse-transcriptase inhibitors, especially zidovudine and didanosine, as well as protease inhibitors have been linked to adverse cardiac function [19–21]. Nevertheless, there is mounting evidence supporting the fact that, globally, ART decreases the incidence of cardiac abnormalities, probably by reducing the rate of coinfections (myocarditis and pericarditis), pulmonary hypertension and consequent dilated cardiomyopathy [10,22]. Results from our study support this hypothesis, as no impairment of cardiac function was found in our ART-treated cohort of children and adolescents, even though newer and more sensitive echocardiographic methods for the early detection of cardiac abnormalities were used.

HIV-infected patients in our study showed slightly lower ejection fraction and fractional shortening, and also slightly greater LV end-systolic diameter. Although statistically significant, these differences lack of clinical relevance, as all parameters remained within normal range except for the four participants that were diagnosed with systolic dysfunction. Furthermore, the findings were not confirmed by the more sensitive methods of tissue Doppler imaging or speckle tracking. Despite the absence of reference values, longitudinal LV strain between -16 to -22% has been previously considered as normal [23]. Normal values for LV rotation/twist angle show variability depending on the technique used and age of the subject, mainly because of a gradual increase in apical rotation with age [24]. However, a peak LV twist angle of less than 10 degrees can be considered normal [25]. In our view, a “white-coat hypertension”-like phenomenon might have contributed to the differences found in load-dependent cardiac measurements, as ejection fraction or fractional shortening. Indeed, parameters unaffected by loading cardiac conditions, as STE-derived myocardial strain, showed no difference at all. Most controls were healthy subjects recruited in a high

school and not used to medical procedures, and thus tended to show higher blood pressure and heart rate, which can definitely influence these parameters by overestimating systolic performance.

Although no clinically significant differences in cardiac function were found between groups, we explored relations between HIV-related variables and echocardiographic parameters. According to the literature, disease severity appears to be the main factor related to abnormalities on cardiac structure and function in adult population [26]. In our study, after multivariate analysis no correlation was found between traditional echocardiographic parameters and detectable viral loads, CD4 counts or CD4 nadir. Our findings are consistent with the last study published by Lipshultz et al. that describe an association between CD4 nadir and cardiac function in the pre-ART era, whereas no association is found in early treated patients [10]. Due to the frequency of viral blips during adolescence, results regarding the effect of viral suppression on cardiac health have to be interpreted with extreme precaution.

Deterioration of standard echocardiographic measures of EF and FS is generally considered a late stage of cardiac dysfunction in clinically asymptomatic patients. In an attempt to overcome this fact, we included in our cardiac evaluation measurements of longitudinal strain and rotational motion analysis, aiming to early detect patients at higher risk for cardiac dysfunction. Except for a tendency towards a minimal increase in ventricular torsion found in HIV-infected subjects that did not reach statistical significance, no differences were found in ventricular function between groups. These findings disagree with the study published in 2012 by Sims et al. [11], including 28 ART-treated HIV-infected children and young adults and matched controls, in which HIV-infected individuals presented mild cardiac impairment missed by conventional echocardiography but detected by cardiac strain analysis. Mean age of subjects included in the mentioned study was 18±4 years [range 7-29], whereas mean age in our cohort is 14±5 [range 2.5-22]. We hypothesize that what might be seen as a small difference in ages may in fact have led to rather big differences in terms of ART history and disease progression, as ART was standardized for prevention of vertical transmission in the late nineties. At that time, the amount of drugs approved to be

used in children varied substantially from one year to another, with great impact on achievement and maintenance of viral suppression. Most participants in our study were treated early and frequently achieved and maintained undetectable viral loads at least until adolescence, and had an overall better disease control compared to their peers born five years before. Nevertheless, as impairment of cardiac function appears progressively, it is also possible that mild abnormalities that are not yet present during adolescence might become apparent in young adults. Whether the development of cardiac dysfunction is related to immunosuppression and worse disease control, or is directly related to the infection itself and thus, just a matter of time, would have to be answered in longitudinal studies.

We present here data from a cross-sectional analysis, so no causal inferences can be made. The rather small sample size of the study obviously limits our ability to measure the contribution of HIV-related variables on cardiac function. Larger studies would potentially allow us to deepen into the associations between ventricular function and particular antiretroviral drugs, which may help to identify optimal combinations in terms of protecting long-term cardiac health. In order to minimize the weight of classical cardiovascular risk factors, controls in our study were enrolled aiming to achieve a group with similar age, sex, BMI and socio-economical background. Along with this, HIV-exposed but uninfected children were included in the study, as they have historically been considered as the optimal control group. However, recent studies suggest that intra-utero exposure to ART, as well as to the virus itself, may impact on cardiac status, and these findings put into question the appropriateness of this population as the optimal reference [10,27]. In order to further confirm our findings, we repeated the analyses restricting the control group to HIV-unexposed subjects, and results were consistent with previous findings. Clearly, recruitment of the ideal control group in terms of cardiovascular risk remains to be a challenge.

While the mechanistic pathways of cardiac dysfunction during HIV infection are further enlighten, we agree that perinatally HIV-infected individuals may benefit from cardiac evaluation for a prompt diagnosis and management of abnormalities. Although the optimal timing of a cardiac study is unclear, according to the findings from the

previously mentioned studies and to the data provided in the present work, we believe that the end of adolescence might be the ideal moment to perform a follow-up echocardiogram. Larger and prospective studies are warranted in order to define the long-term cardiac health of early and effectively treated perinatally HIV-infected children.

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HIV-infected Individuals with Low CD4/CD8 Ratio despite Effective Antiretroviral Therapy Exhibit Altered T-cell Subsets, Heightened CD8+ T-cell Activation, and Increased Risk of Non-AIDS Morbidity and Mortality

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Running title: Low CD4/CD8 ratio during treated HIV infection

ABSTRACT

A low CD4/CD8 ratio in elderly HIV-uninfected adults is associated with increased morbidity and mortality. A subset of HIV-infected adults receiving effective antiretroviral therapy (ART) fails to normalize this ratio, even after they achieve normal CD4+ T-cell counts. The immunologic and clinical characteristics of this clinical phenotype remain undefined. Using data from four distinct clinical cohorts and two clinical trials, we show that a low CD4/CD8 ratio in HIV-infected adults during otherwise effective ART (after CD4 count recovery above 500 cells/mm^3) is associated with a number of immunological abnormalities, including a skewed T-cell phenotype from naïve towards terminally differentiated CD8+ T-cells, higher levels of CD8+ T-cell activation (HLADR+CD38+) and senescence (CD28- and CD57+CD28-), and higher kynurenine/tryptophan ratio –a marker of indoleamine-dioxygenase-1 induction–. Changes in the peripheral CD4/CD8 ratio are also reflective of changes in gut mucosa, but not in lymph nodes. In a longitudinal study, individuals who initiated ART within six months of infection had greater CD4/CD8 ratio increase compared to later initiators (>2 years). After controlling for age, gender, ART duration, nadir and CD4 count, the CD4/CD8 ratio predicted increased risk of morbidity and mortality. Hence, a persistently low CD4/CD8 ratio during otherwise effective ART is associated with increased innate and adaptive immune activation, an immunosenescent phenotype, and higher risk of morbidity/mortality. This ratio may prove useful in monitoring response to ART and could identify a unique subset of individuals needed of novel therapeutic interventions.

Keywords: HIV; CD4/CD8 ratio; CD4+ T-cell; CD8+ T-cells; inflammation; innate immunity; adaptive immunity; immune activation, immunosenescence; indoleamine-dioxygenase-1; predictors; antiretroviral therapy; non-AIDS related events; morbidity; mortality

AUTHOR SUMMARY

The CD4/CD8 ratio, a hallmark of the collection of T-cell defects related to aging –"immunosenescence"- and a predictor of mortality in the general population, often fails to normalize in an important proportion of HIV-infected individuals with adequate CD4+ T-cell

recovery after ART initiation. However, the immunological and clinical characteristics of this clinical phenotype have not been elucidated. Herein we show that during treated HIV infection, expansion of CD8+ T-cells, reflected as a low CD4/CD8 ratio, identifies a subgroup of individuals with a number of immunological abnormalities and a poor prognosis. These subjects exhibit increased innate and adaptive immune activation, an immunosenescent phenotype, CD4+ and CD8+ imbalance in the gut mucosa and higher risk of morbidity and mortality. In contrast, those who normalize the CD4/CD8 ratio have traits of a healthy immune system. We observed that early ART initiation might contribute to more rapid and robust CD4/CD8 ratio normalization compared to later initiation. Hence, the CD4/CD8 ratio might help to further discriminate the risk of disease progression of successfully treated HIV-infected individuals, and a successful response to ART may require both normalization of the peripheral CD4+ T cell count and the ratio of CD4+ to CD8+ T-cell counts.

INTRODUCTION

It is now anticipated that HIV-infected adults who have access to modern antiretroviral therapy (ART) should be able to suppress HIV replication indefinitely. Although treatment-mediated increases in the peripheral CD4 count are associated with reduced morbidity and mortality, compared to age-matched individuals without HIV infection, those on ART have a higher risk of morbidity and mortality. Compared to age-matched individuals without HIV infection, for example, those on ART have a higher risk of morbidity and mortality. This risk is predicted in part by the on therapy CD4 count, although achieving an apparent normal CD4 count may not fully restore health [1–5]. Indeed, recent large cohort studies have emphasized that even those treated patients with CD4+ T cell counts above 500 cells/mm^3 have a higher risk of AIDS and non-AIDS morbidities and mortality compared to people with HIV or much lower CD4+ T cell counts [6,7]. The decreased life expectancy during ART-mediated viral suppression has largely been associated with an increase in risk of non-AIDS-morbidity, a term that entails a group of conditions generally associated with aging, including aging-associated malignancies and cardiovascular, renal, liver, neurologic, and bone disease [4,8,9].

While the mechanisms driving the increased burden of aging-associated disease in HIV-infected individuals are not fully understood, an emerging body of evidence suggests that persistent innate and adaptive immune dysfunction and/or activation are major risk factors [10–13]. Many of the immunologic abnormalities that persist during therapy are similar to those observed in the elderly, raising the hypothesis that it is immunosenescence, the aging-associated decline in immunocompetence, that ultimately leads to disease progression and adverse outcomes [14–17]. Markers of innate immune activation [e.g. interleukin (IL)-6, high-sensitivity C reactive protein (hs-CRP) and soluble CD14 (sCD14)], coagulation (fibrinogen, D-dimers), bacterial translocation (lipopolysaccharide), and T-cell activation (HLADR and CD38 co-expression) are elevated despite effective ART and in many studies are associated with subsequent morbidity and mortality, even after adjustment for CD4+ T-cell count [18–22]. This fact strongly suggests that an underlying mechanism not captured by CD4+ T-cell count and HIV replication might be contributing to disease progression.

The importance of CD4 counts as a strong predictor of opportunistic infections and non-AIDS events has been widely investigated, but little attention has been paid to the prognostic significance of CD8 counts. During untreated HIV infection, CD8 counts increase as CD4 counts decline –the so-called "blinded T-cell homeostasis" [23]. During ART-mediated viral suppression, some individuals achieving CD4 counts ≥500 cells/mm^3 experience a simultaneous decline in CD8 counts, leading to normalization of the CD4/CD8 ratio to levels above 1.0. Others, however, maintain high levels of circulating CD8+ T-cells, and hence a persistently low CD4/CD8 ratio [24]. The biological and clinical significance of this imbalance in CD4+ and CD8+ T-cell homeostasis remains obscure. Among elderly HIV-uninfected adults, inversion of the CD4/CD8 ratio (<1.0) has been considered a surrogate of immunosenescence and a predictor of all-cause mortality [25–29]. In the setting of untreated HIV infection, the CD4/CD8 ratio predicts time to AIDS [30] and is associated with pre-ART CD4 and CD8 counts [24,31]. During ART, this ratio appears to correlate with increased markers of T-cell activation and senescence [32,33], of aging-associated disease,[34,35] and to predict non-AIDS morbidity and mortality [36].

We hypothesized that among ART-treated HIV-infected individuals with CD4 counts ≥500 cells/mm^3, expansion of CD8+ T-cells, reflected as a low CD4/CD8 ratio, may identify individuals with persistent innate and adaptive immune activation at greater risk of serious non-AIDS events. Since early ART initiation has been shown to reduce levels of T-cell activation, we hypothesized that earlier ART initiation might also accelerate the rate of CD4/CD8 ratio normalization.

METHODS

Subjects

Study subjects were sampled from four cohorts and two clinical trials:

1) SCOPE: a clinic-based cohort of over 1500 chronically HIV-infected participants and HIV-uninfected controls in San Francisco; 2) the Study of the Ocular Complications of AIDS (SOCA): a multicenter cohort of over 2200 HIV-infected participants who initiated ART with an AIDS diagnosis; 3) OPTIONS: a clinic-based cohort of participants diagnosed during acute/early HIV infection, previously described[37]; 4) the Madrid cohort: a clinic-

based cohort of 2400 ART-treated individuals, 130 of whom developed serious non-AIDS events. 5) the raltegravir (NCT00631449) [38] and maraviroc (NCT00735072) [39] ART intensification randomized, placebo-controlled, clinical trials in HIV-infected individuals. Additional information on the cohorts and the clinical trials can be found in **Appendix 1**.

Ethics statement

These studies were approved by the UCSF Committee on Human Research or by the Ethics Committee of the University Hospital Ramón y Cajal. All participants were adults and provided written informed consent in accordance with the Declaration of Helsinki.

Measurements

T-cell immunophenotyping was performed on cryopreserved peripheral blood mononuclear cells (PBMC), in fresh lymph node mononuclear cells (LNMC) from inguinal biopsies obtained from HIV-infected volunteers under ART-mediated viral suppression, and in mucosal mononuclear cells (MMC) obtained from rectal biopsies in the maraviroc and raltegravir studies, as previously described [38–40]. Fresh inguinal lymph nodes were biopsied were minced and strained through a 70 micron filter to created a single cell suspension of LNMC. MMC were isolated from biopsy specimens using a protocol optimized for lymphocyte viability and yield [41]. Cells were thawed, washed, stained with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen) to exclude non-viable cells and stained with fluorescently-conjugated monoclonal antibodies (recognizing CD3, CD4, CD8, HLADR, CD38, CD27, CD28, CCR5, CCR7, CD45, PD1 for PBMC and CD3, CD4 and CD8 for LNMC and MMC; see **Appendix 2**). Cells were then fixed in 0.5% formaldehyde and ≥250,000 were analyzed on a BD LSR II Flow cytometer (BD Biosciences) using FlowJo (Tree Star) to determine the proportion of CD4+ and CD8+ T-cells expressing each of the T-cell markers. Combinations of markers were calculated in FlowJo, using the Boolean gate function. We determined in PBMC the T-cell maturation subsets, defined as naïve (T_N, CD45RA+CCR7+CD27+CD28+), central memory (T_{CM}, CD45RA-CCR7+CD27+CD28+), transitional memory (T_{TR}, CD45RA-CCR7-CD27+CD28-), effector memory (T_{EM}, CD45RA-CCR7-CD28-CD27-), and terminally differentiated (T_{EMRA}, CD45RA+CCR7-CD27-CD28-), as well as the phenotypes of activated/senescent CD8+ T-cells (HLADR+CD38+, CD28-, CD57+CD28-, and PD-1+), and the proportion of CD28-CD8+

T-cells expressing CD57, which has been recently described as a unique CD8+ T-cell defect in HIV that appears to be distinct from the classical immunosenescent phenotype found with aging and that predicts mortality [42]. In LNMC and MMC we determined the % of CD4+ and CD8+ T-cells. Additional information is provided in the supplemental material.

Cryopreserved plasma was assessed by immunoassay for IL-6 (R&D Systems), sCD14 (R&D Systems), hs-CRP (CardioPhase hs-CRP assay, Siemens), D-dimer (DiagnosticaStago), intestinal fatty acid binding protein (I-FABP, Cell Sciences) and zonulin-1 (ALPCO) levels. Plasma tryptophan and kynurenine levels were measured by high performance liquid chromatography tandem mass spectroscopy [43], and the activity of indoleamine 2,3-dioxygenase-1 (IDO-1) was assessed as the plasma kynurenine to tryptophan (KT) ratio. Chronic asymptomatic CMV infection was confirmed by a positive CMV IgG titer and for a subset of HIV-infected participants without available CMV serology, >0.1% pp65/IE-specific IFN-γ+ CD8+ T-cell responses by cytokine flow cytometry (ten-fold increase over limit of detection) as previously described [44].

Statistical Methods

Cross-sectional pairwise comparisons between groups were performed using Wilcoxon rank sum tests. Since a "normal" CD4/CD8 ratio remains poorly defined, for the between-group comparisons of T-cell subsets and percentages of activated/senescent CD8+ T-cells, we classified individuals according to the lowest quartile (≤0.4) and highest quartile (≥1.0) of SCOPE participants with ≥500 CD4+ T-cells/mm^3. A CD4/CD8 ratio ≤0.4 has been defined previously as the best cutoff that may predict serious non-AIDS events in well-treated HIV-infected patients [36], and 1.0 has been suggested in the general population as the cutoff for the "immune risk profile" associated with immunosenescence and mortality [26,45].

To analyze the association between the CD4/CD8 ratio and the KT ratio in SOCA, we fitted a linear regression model being the dependent variable the CD4/CD8 ratio and the KT ratio the explanatory variable, adjusting the model by age, gender, time under viral suppression and CD4 nadir. To evaluate the relative contribution of the CD4+ and

CD8+ T-cells to this association, we also fitted a model with both CD4+ and CD8+ T-cells adjusting for the same covariates.

We analyzed the correlations between the CD4/CD8 ratio in blood, with the ratio in lymph nodes and in GALT. For the GALT CD4/CD8 ratio measured in the MVC and RAL studies, we used only baseline measurements (before ART intensification). Since a different panel of antibodies was used for each study for flow-cytometry analysis, we fitted a linear regression analysis adjusting by the source study.

We analyzed the impact of early ART initiation on the CD4/CD8 ratio in the OPTIONS cohort among recently HIV-infected participants, focusing on those who either started ART within six months of infection (early ART) or who deferred therapy for at least two years (later ART).[37] Longitudinal changes in CD4 and CD8 counts and in the CD4/CD8 ratio were assessed using linear mixed models with random intercepts. Age, gender, and pre-ART CD4 counts were included in multivariate analyses as fixed-effects. Interaction terms were created to assess whether these changes over time differed significantly between the early and later ART initiators. Changes in slopes before and after ART time points were assessed using linear splines.

We used data from the Madrid and SOCA cohorts to evaluate whether the CD4/CD8 ratio might be a marker of non-AIDS-related morbidity and mortality, respectively. In the nested case-control analysis in the Madrid cohort, cases who developed serious non-AIDS events and had ≥500 CD4+ T-cells/mm^3, were each matched to one controls by age, sex, nadir CD4, and proximal CD4 counts (N=66). In the nested case-control study of immunological predictors of mortality in SOCA, cases with non-accidental death who had PBMC and plasma samples available within 18 months of death with confirmed plasma HIV RNA levels <400 copies/ml were each matched to two controls by age, gender, duration of viral suppression, history of CMV retinitis, and nadir CD4 (N=183). We used conditional logistic regression to evaluate the CD4/CD8 ratio as a predictor of non-AIDS morbidity/mortality. Continuous variables in multivariate models were log-transformed when necessary to satisfy model assumptions.

RESULTS

Low CD4/CD8 ratio during effective ART is associated with prominent immunosenescence

We first analyzed the correlations between the CD4/CD8 ratio, T-cell maturation subsets and different phenotypes of activated T-cells among ART-suppressed individuals. The general characteristics of this population are summarized in **Table S1**. Higher CD4/CD8 ratio was correlated with higher frequencies of T_N (Rho=0.35, P=0.005), T_{CM} (Rho=0.272, P=0.03), and T_{TR} CD8+ T-cells (Rho=0.25, P=0.05), but lower frequencies of T_{EM} (Rho=-0.37,P=0.003) and T_{EMRA} (Rho=-0.26, P=0.024) CD8+ T-cells. Overall, the CD4/CD8 ratio was more strongly associated with the proportions of T-cell maturation subsets and proportions of activated CD8+ T-cell phenotypes than were the CD4 or CD8 counts (see **Table 1**).

To underline the association between a low CD4/CD8 ratio and persistent T-cell abnormalities during effective ART, we compared ART-suppressed HIV-infected individuals with ≥500 CD4+ T-cells/mm^3 (N=67) in the lowest (≤0.4) versus highest (≥1) quartiles of CD4/CD8 ratio and healthy controls CMV+ (N=15) (see **Table S1** for the clinical characteristics of each group and **Figures 1,2 and S1** for the between-group comparisons). Median CD8 counts were markedly higher among those with low versus high CD4/CD8 ratio (1964 cells/mm^3 vs. 696 cells/mm^3, respectively). In subjects with low CD4/CD8 ratio, the higher absolute CD8 counts led to an apparent mismatch in the proportions and absolute counts of early-differentiated CD8+ T-cell maturation subsets **(Figure 1*A*-*B*)**, but then for late-differentiated memory CD8+ T-cells, demonstrated increased proportions and absolute counts of T_{EM} and T_{EMRA} cells. In contrast, ART-suppressed participants with a high CD4/CD8 ratio did not demonstrate any differences in the proportions of CD8+ T-cell maturation subsets compared to healthy controls. ART-suppressed individuals with a low CD4/CD8 ratio also had higher proportions of activated (HLADR+CD38+) and "senescent" (CD28- and CD28-CD57+) CD8+ T-cells, in comparison to participants with a high CD4/CD8 ratio who had levels comparable to controls (**Figures 2*A*-*B*)**. However, both ART-suppressed (CD4≥500 T-cells/mm^3) participants with low and high CD4/CD8 ratios, had lower proportions of CD28-CD8+ T-

cells expressing CD57 compared to healthy controls, consistent with prior data **(Figure 2A)** [42].

The CD4/CD8 ratio as a marker of innate immune dysfunction

To explore the potential mechanisms driving the expansion of late-memory T-cells in subjects with low CD4/CD8 ratio despite effective ART we used data from the SOCA cohort (general characteristics summarized in **Table S2**). We analyzed the correlations between CD4 and CD8 counts and the CD4/CD8 ratio and different markers of innate immune activation and epithelial integrity (**Table 2**). We observed across all subjects significant inverse correlations between the CD4/CD8 ratio and hs-CRP, IL-6, sCD14 and the KT ratio. However, in the subgroup of subjects with ≥500 CD4+ T-cells/mm^3, only the KT ratio remained significantly correlated with the CD4/CD8 ratio (Rho=-0.30, P=0.041) (**Figure 3*A*)**. This association was confirmed in a linear regression analysis adjusted for age, gender and cumulative ART exposure (Beta=-0.72, P=0.009), where for each 10% increase in the CD4/CD8 ratio there was a 7% decrease in the KT ratio. The CD4/CD8 ratio performed better as a predictor of the KT ratio than the CD4+ or CD8+ T-cell counts in a similar model (see **Table 3**). Since subjects with low ratio and ≥500 CD4+ T-cells/mm^3 were enriched for CD28-CD8+ T-cells and also showed increased IDO induction, we hypothesized that a potential underlying mechanism driving expansion of CD28-CD8+ T-cells might be IDO induction, and we found a positive correlation between these two variables (Rho=0.50, P <0.001). These results indicate that the CD4/CD8 ratio predicts better the degree of IDO induction than the CD4 or CD8 counts individually, which is especially evident above the threshold of 500 CD4+ T-cells/mm^3, and that increased IDO induction might be driving expansion of CD28-CD8+ T-cells and a low CD4/CD8 ratio.

Correlations between the CD4/CD8 ratio in blood and lymphoid tissues

A persistently low CD4/CD8 count ratio in peripheral blood might conceivably be the result of differential redistribution of CD4+ and CD8+ T-cells out of lymphoid tissues, but to our knowledge, no study has assessed whether the CD4/CD8 ratio in peripheral blood is reflective of the CD4/CD8 ratio in tissues. To address this question, we analyzed the correlations of the CD4/CD8 ratio in blood with the CD4/CD8 ratio in lymph node and in GALT (see **Table S3**). Using data from 10 individuals on ART, no significant correlation between the CD4/CD8 ratio in blood and in lymph nodes was observed (Rho=-0.07, P=0.855) (**Figure 4*A***). For the correlation between the CD4/CD8 ratio in GALT and in blood (**Figure 4*B***), we used data from 32 individuals on ART, and the CD4/CD8 ratio in blood strongly correlated with that in rectal mucosa (Rho=0.68, P<0.001 and Beta=0.69, P<0.001). These results indicate that while correlated, the imbalance between CD4+ and CD8+ T-cells is less marked in blood than in GALT.

Impact of timing of ART initiation on the dynamics of circulating CD4+ T-cells, CD8+ T-cells and CD4/CD8 ratio

We next examined the extent to which persistent abnormalities in the CD4/CD8 ratio were associated with later vs. earlier initiation of ART using the described OPTIONS cohort of recently infected adults who started therapy during the first six months of their infection (early ART) or after two years of untreated infection (later ART) (see **Table S4**) [37]. At the time of their diagnosis, median CD4/CD8 ratio was significantly lower in recently HIV-infected individuals compared to the HIV-uninfected group (**Figure 5*A***, all baseline comparisons, P<0.05). The later ART group remained untreated for a median of 3 years. The CD4 count declined by 274 cells/mm^3, the median CD8 count increased by 125 cells/mm^3, and the CD4/CD8 ratio decreased from 0.76 to 0.38.

After one year of ART, both early and late ART initiators experienced a substantial increase in CD4 count (**Figures 5*B*-*C*)**. However, while early ART subjects also showed a substantial decline in CD8+ T-cells after one year of ART, in the later ART group the CD8 count decrease only became statistically significant after a median follow-up of three years (**Figures 5*D*-*E*).** After one year of ART, early treated patients showed significantly

higher median CD4/CD8 ratio (1.0 vs. 0.57, P<0.001) and had fourfold-increased odds of CD4/CD8 ratio normalization during follow-up (OR, 3.6; 95% CI, 1.2, 10.8; P=0.022). The greater effect of early ART compared to later ART on the CD4/CD8 ratio remained statistically significant after adjustment by age, gender, and baseline CD4+ T-cell counts in the mixed-effects linear model (**Figures 5*F-G*).** The mean CD4/CD8 ratio change predicted by the model was significantly higher among early ART initiators compared to later initiators after one year of ART (+0.44 vs. +0.25, respectively, P<0.001), and after a median of 3 years of ART (+0.61 vs. +0.49, respectively, P<0.001). In summary, early ART initiators experienced a faster CD4/CD8 ratio increase and reached a higher CD4/CD8 ratios after a median of 3 years of ART, which was primarily driven by changes in the CD4 counts and, to a lesser degree, by changes in the CD8 counts.

Low CD4/CD8 ratio despite effective ART predicted disease progression beyond the CD4+ and CD8+ T-cell counts

Lastly, we hypothesized that the prognostic importance of the CD4/CD8 ratio might depend upon the relative predictive contribution of both CD4 and CD8 counts. We used the nested case-control study in the Madrid cohort to evaluate in ART-suppressed subjects with ≥500 CD4+ T-cells/mm^3 whether a low CD4/CD8 ratio might be a predictor risk of serious non-AIDS events. We used the nested case-control study in the Madrid cohort to evaluate in ART-suppressed subjects with ≥500 CD4+ T-cells/mm^3 whether a low CD4/CD8 ratio might be a predictor risk of serious non-AIDS events. A sample of 33 cases with CD4 counts ≥500 cells/mm^3 was matched to 33 controls by age, gender, nadir CD4 and proximal CD4+ T-cell counts (see **Table S5** for the general characteristics of the study population and **Table S6** for the description of non-AIDS events). We observed that both the CD4/CD8 ratio and CD8 count independently predicted the risk of non-AIDS events, with the coefficient of the CD4/CD8 ratio significantly higher **(see Table 4)**. After controlling for age, gender, ART duration, nadir and proximal CD4 count, each 10% decrease in the CD4/CD8 ratio and each 10% increase in the CD8+ T-cell counts were associated with 48% and 22% higher odds of serious non-AIDS events, respectively.

To assess the relationships with mortality, we used SOCA cohort –a group of participants with more advanced HIV infection of 62 cases of death matched by age, gender, nadir CD4+ T-cell count, and duration of viral suppression with 121 controls–

(see **Tables S2** and **S6**). We observed that both the CD4/CD8 ratio and CD4+ T-cells, but not CD8+ T-cells, were independent predictors of mortality –for each 10% increase in the CD4/CD8 ratio or in CD4+ T-cells and there was a 15% and 13% decrease in the risk of death, respectively **(see Table 4)**.

DISCUSSION

Combining the data from four clinical cohorts and two clinical trials, we demonstrate here that a substantial subset of ART-suppressed HIV-infected adults who have achieved virologic suppression and a normalized peripheral CD4 count (≥500 cells/mm^3) have persistently elevated CD8 counts and a low CD4/CD8 ratio. This ratio, in turn, is correlated with markers of T-cell activation and innate immune activation and with the presence of a previously described immunosenescent phenotype (i.e., low naïve T-cell frequencies and increased frequency of terminally differentiated CD57 expressing cells). This imbalance in T-cell homeostasis measured in blood is also present in GALT. Although early ART (<6 month after HIV infection) is associated with more rapid normalization of the CD4/CD8 ratio, an abnormal ratio persists even in these aggressively treated individuals. Among well-treated individuals with high CD4 count, a low ratio was an independent predictor of serious non-AIDS events and mortality. Collectively, these results suggest that a persistently low CD4/CD8 ratio during ART may be a marker of persistent immune dysfunction and inflammation, and that monitoring of this ratio—which can be readily done in most clinics with current assays—may be clinically useful. A truly successful response to ART may require both normalization of the peripheral CD4 count and the CD4/CD8 ratio.

The immunologic profile of the individuals in our cohorts with a persistently low CD4/CD8 ratio despite high CD4 counts is similar to that observed in the very old. T-cell "immunosenescence" is generally defined as a low naïve/memory T-cell ratio, expansion of CMV-specific CD8+ T-cells, enrichment for CD28- and PD-1+ T-cells, increased CRP and IL-6 levels, reduced T-cell telomere lengths and a low CD4/CD8 ratio [46]. Since untreated HIV infection is associated with each of these immunologic characteristics, it has been proposed that HIV might accelerate the aging of human immune system [14,47–49]. The extent to which successful ART reverses these HIV-induced immune

changes is currently the subject of intense investigation.[31] Our data argue against the existence of a "blinded" T-cell homeostasis during long-term ART, and indicate that the biology of CD4+ T-cell counts may be different than that of CD8+ T-cell counts, in terms of impacts of immunodeficiency and chronic inflammation, as suggested by others [50–52].

We also studied a number of related markers of immunosenescence, using as a comparator group HIV-uninfected adults who were infected with CMV (as nearly all HIV-infected subjects are co-infected with this virus). We found that HIV-infected subjects who achieved CD4/CD8 ratio normalization during ART demonstrated traits of a nearly healthy immune system, with T-cell maturation subsets and levels of CD8+ T-cell activation/senescence comparable to those observed in healthy subjects. In contrast, a CD4/CD8 below ≤0.4 identified individuals with prominent features of immunosenescence despite CD4+ T-cell recovery, including reduction of the CD8+ naïve T-cell compartment, enrichment for T_{EM} and T_{EMRA} CD8+ cells, and increased levels of CD8+ T-cell activation (HLADR+CD38+ T-cells) and senescence (CD28- and CD57+CD28- T-cells). Expansion of CD28-CD8+ T-cells is a hallmark of replicative senescence, a term describing the phenomenon in which long-lived cells that have undergone multiple rounds of proliferation, show telomere shortening and hence, limited proliferative potential [53,54].

The CD4/CD8 ratio inversely correlated with several markers of innate immune activation (sCD14, hs-CRP, IL-6) and with a biomarker of IDO induction (KT ratio), but only the association with the KT ratio remained significant in the subgroup of individuals with ≥500 CD4 T-cells/mm^3, perhaps because of the very high biologic variability in these assays. IDO is induced by HIV in activated dendritic cells and monocytes, and catabolizes tryptophan into kynurenine and other immunologically active catabolites that suppress T-cell proliferation and/or differentiation. It has been argued that induction of IDO may represent a critical initiating event that results in inversion of the TH17/T regulatory balance, loss of epithelial barrier integrity and thereby maintenance of a chronic inflammatory state in progressive HIV disease [43,55]. Since the KT ratio correlated well with CD28-CD8+ T-cells, our data suggest that increased IDO activity may be contributing to the replicative CD8+ T-cell senescence observed in ART treated subjects with low

CD4/CD8 ratio. Alternatively, the accumulation of CD8+ T-cells might be the cause of increased IDO activity, as a consequence of greater IFN-Ɣ production.

We observed that a low CD4/CD8 ratio is a predictor of serious non-AIDS events and mortality among treated individuals with ≥500 CD4+ T-cells/mm^3 in the Madrid-based cohort, with much of the association driven by the CD8 counts. This observation expands upon our previous findings in this cohort, demonstrating that an increased risk of non-AIDS defining neoplasias, cardiovascular events, and associated mortality is independently predicted by the CD4/CD8 ratio [36]. We observed an association between the ratio and mortality in the entire SOCA cohort, but in this cohort selected based on low CD4 nadir and which had a lower range of CD4 counts, the association depended upon the CD4 counts. These two analyses suggest that while the CD4 counts predict mortality in those individuals with low CD4 counts, this effect becomes subtle and difficult to detect in those with high CD4 counts, where high CD8 counts might drive adverse outcomes. In both scenarios, it seems that the CD4/CD8 ratio retains the predictive importance of both CD4+ and CD8+ T-cells.

As suggested by the present study, the group of ART-treated HIV-infected individuals with CD4+ T-cell counts above 500 cells/mm^3 represents a clinical spectrum of individuals, ranging from those with an immune system that is abnormal (e.g., with a CD4/CD8 ratio ≤0.4) to those with an apparently normal immune system (e.g., with a CD4/CD8 ratio ≥1), a finding that might serve to explain the discrepancies in cohort studies addressing morbidity and mortality among successfully treated HIV-infected individuals [4,6–9,56–58]. We suggest that the CD4/CD8 ratio might help to further discriminate the risk of disease progression of successfully treated HIV-infected individuals.

Mechanistically, we imagine that the maintenance of an abnormally high level of circulating CD8+ T-cells could be the result of increased proliferation, decreased death, and/or changes in the rate at which these cells move between organized lymphoid structures and the peripheral blood. Although we have no evidence for increased levels of proliferation (e.g., as measured by incorporation of stable isotopes or expression of Ki67), previous studies have shown that the T_{EMRA} subset has a longer lifespan in the setting of untreated HIV disease [59], a property that may well be found in treated individuals with a low CD4/CD8 ratio. There are also data showing that progressive as

well as treated HIV disease is associated with collagen deposition and loss of the fibroblastic reticular cell network within lymphoid tissue, particularly in the context of inflammation [60,61], and such structural changes might result in the presence of an unusually large proportion of circulating CD8+ T-cells. Since each of these pathologic changes is associated with a high level of inflammation, it follows that resolution of inflammation in the effectively treated individual with a low CD4/CD8 ratio might result in normalization of the ratio over time.

There are limitations to the current study that deserve mention. First, for the analysis of the correlation between the CD4/CD8 ratio in blood and GALT we used data from two clinical trials involving individuals with suboptimal CD4+ T-cell recovery; hence, further studies in individuals with CD4+ T-cell recovery above 500 cells/mm^3 are needed to assess whether a low CD4/CD8 ratio reflects poor GALT immune reconstitution in these subjects. Secondly, in the subset of individuals above 500 cells/mm^3, there were only 8 episodes of non-AIDS related death in the Madrid cohort, and 16 in SOCA cohort, which prevented us from performing mortality analysis in this subgroup. However, in a previous study in the Madrid cohort not restricted by CD4+ T-cell counts and analyzing the risk of cardiovascular events and non-AIDS defining neoplasias in more than 400 ART-treated HIV-infected individuals, a low CD4/CD8 ratio predicted non-AIDS-related morbidity and mortality [36] and the association is also supported by the mortality analysis in SOCA cohort, as wells as from data in the general population [25,45].

Our results have potential clinical implications for novel therapeutic strategies targeting immune dysfunction in chronically treated HIV-infected individuals, in particular those with persistent expansion of CD8+ T-cells despite adequate CD4+ T-cell recovery. This CD4/CD8 ratio may be useful in monitoring response to therapies aimed at reducing residual immune activation, and given that prior studies have also reported that a low CD4/CD8 ratio is associated with increased markers of HIV persistence [62,63], subjects with a high CD4/CD8 ratio may be useful targeted candidates for HIV eradication trials. Finally, ART-suppressed HIV-infected individuals who do not have an increase in the CD4/CD8 ratio might benefit from screening programs or aggressive management of concomitant risk factors for aging-associated disease.

In summary, a low CD4/CD8 ratio among ART-treated HIV-infected individuals achieving CD4+ T-cell counts above 500 cells/mm^3 may define a new clinical phenotype

of individuals with higher levels of CD8+ T-cell activation and senescence, depletion of naïve and enrichment for T_{EMRA} cells, increased IDO activity and higher risk morbidity and mortality. Early ART initiation may contribute to more rapid and robust CD4/CD8 ratio normalization, and the CD4/CD8 ratio may be a useful clinical endpoint to be used in evaluating novel therapies for ongoing immune dysfunction during treated infection and for HIV eradication.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the participation of all the study participants who contributed to this work as well as the clinical research staff of the SCOPE, OPTIONS, SOCA, and Madrid cohorts who made this research possible.

TABLES

Table 1. CD4+ T-cell counts, CD8+ T-cell counts and CD4/CD8 ratio correlations with the percentage of T-cell maturation subsets and T-cell activation phenotypes in HIV-infected participants in SCOPE cohort.

	CD4+ T cell count Rho (P value)	CD8+ T cell count Rho (P value)	CD4/CD8 ratio Rho (P value)
ALL SUBJECTS (n=95)			
%CD4+ T-cells			
Maturational subsets			
Naïve	**0.384 (<0.001)**	-0.042 (0.695)	**-0.150 (0.001)**
Tcm	0.179 (0.093)	0.05 (0.612)	-0.179 (0.090)
Ttm	**-0.395 (<0.001)**	0.052 (0.626)	**-0.364 (<0.001)**
Tem	-0.033 (0.754)	-0.068 (0.525)	0.028 (0.797)
Temra	0.118 (0.271)	0.068 (0.530)	0.035 (0.746)
Activation phenotypes			
HLADR+CD38+	**-0.577 (<0.001)**	0.008 (0.937)	**-0.410 (<0.001)**
CD28-CD57+	**-0.209 (0.048)**	-0.004 (0.968)	-0.149 (0.159)
PD1+	**-0.565 (<0.001)**	-0.037 (0.731)	**-0.375 (<0.001)**
%CD8+ T cells			
Maturational subsets			
Naïve	**0.324 (0.002)**	**-0.252 (0.016)**	**0.437 (<0.001)**
T_{CM}	0.011 (0.918)	-0.159 (0.131)	0.123 (0.245)
T_{TM}	0.023 (0.824)	-0.072 (0.501)	0.072 (0.499)
T_{EM}	-0.167 (0.106)	**0.319 (0.002)**	**-0.379 (<0.001)**
T_{EMRA}	**-0.185 (0.007)**	0.167 (0.112)	**-0.297 (0.004)**
Activation Phenotypes			
HLADR+CD38+	**-0.301 (0.003)**	-0.159 (0.133)	**-0.324 (0.002)**
CD28-CD57+	-0.022 (0.838)	0.180 (0.088)	-0.156 (0.140)
CD28-	-0.234 (0.026)	**0.268 (0.010)**	**-0.381 (<0.001)**
%CD57+of CD28-	0.177 (0.094)	-0.082 (0.441)	0.203 (0.054)
PD1+	-0.029 (0.787)	-0.022 (0.831)	0.026 (0.807)
SUBJECTS WITH CD4≥500 cells/mm^3			
%CD4+ T cells			
Maturational subsets			
Naïve	-0.052 (0.686)	-0.130 (0.326)	0.114 (0.378)
T_{CM}	0.056 (0.665)	-0.122 (0.344)	0.134 (0.380)
T_{TM}	0.06 (0.637)	0.088 (0.494)	-0.025 (0.847)

T_{EM}	-0.065 (0.615)	0.072 (0.580)	-0.105 (0.415)
T_{EMRA}	0.039 (0.762)	0.139 (0.281)	0.013 (0.923)
Activation phenotypes			
HLADR+CD38+	-0.171 (0.179)	0.113 (0.380)	-0.148 (0.247)
CD28-CD57+	0.098 (0.450)	0.071 (0.582)	-0.062 (0.633)
PD1+	-0.133 (0.299)	-0.052 (0.685)	0.016 (0.890)
%CD8+ T cells			
Maturational subsets			
Naïve	-0.053 (0.681)	**-0.383 (0.002)**	**0.347 (0.005)**
T_{CM}	0.041 (0.744)	**-0.264 (0.004)**	**0.272 (0.031)**
T_{TM}	0.158 (0.216)	-0.186 (0.144)	**0.249 (0.049)**
T_{EM}	-0.052 (0.680)	**0.376 (0.002)**	**-0.372 (0.003)**
T_{EMRA}	-0.032 (0.803)	**0.376 (0.002)**	**-0.372 (0.003)**
Activation Phenotypes			
HLADR+CD38+	-0.031 (0.773)	**0.273 (0.031)**	-0.234 (0.062)
CD28-CD57+	0.048 (0.7112)	**0.362 (0.004)**	**-0.323 (0.009)**
CD28-	-0.002 (0.986)	**0.453 (0.002)**	**-0.426 (<0.001)**
%CD57+of CD28-	0.096 (0.455)	0.001 (0.995)	0.028 (0.828)
PD1+	-0.002 (0.984)	-0.105 (0.411)	0.148 (0.284)

"%CD57 of CD28-" refers to the percentage of CD28-CD8+ T-cells expressing CD57

Table 2. Correlations with biomarkers of innate immune activation and epithelial integrity is SOCA cohort

All subjects (n=192)	CD4 Rho (P value)	CD8 Rho (P value)	CD4/CD8 ratio Rho (P value)
KT ratio	**-0.225 (0.003)**	0.127 (0.095)	**-0.336 (<0.001)**
sCD14	**-0.228 (0.002)**	0.028 (0.708)	**-0.232 (0.002)**
hs-CRP	**-0.148 (0.049)**	-0.071 (0.345)	**-0.152 (0.043)**
D-dimers	-0.139 (0.063)	-0.05 (0.469)	-0.141 (0.060)
Interleukin-6	**-0.158 (0.035)**	-0.035 (0.639)	**-0.162 (0.031)**
IFABP	-0.138 (0.067)	0.015 (0.841)	-0.146 (0.052)
Zonulin	0.111 (0.142)	-0.048 (0.523)	0.108 (0.155)
Subjects with CD4 ≥500 cells/mm^3 (n=49)			
KT ratio	-0.209 (0.163)	0.252 (0.091)	**-0.298 (0.041)**
sCD14	**-0.394 (0.007)**	-0.011 (0.943)	-0.155 (0.303)
hs-CRP	-0.063 (0.673)	-0.023 (0.876)	-0.039 (0.799)
D-dimers	-0.004 (0.980)	-0.095 (0.537)	0.015 (0.919)
Interleukin-6	-0.152 (0.313)	-0.097 (0.521)	-0.012 (0.930)
IFABP	-0.144 (0.337)	0.144 (0.338)	-0.223 (0.134)
Zonulin	0.066 (0.664)	-0.048 (0.526)	-0.087 (0.569)

Table 3. Multivariate linear regression analysis: associations of the KT ratio (dependent variable) with CD4+ and CD8+ T-cells, and the CD4/CD8 ratio (independent variables) in SOCA cohort

	Beta	Std. error	P value
CD4+ T-cells			
All subjects	-0.366	0.095	<0.001
Subjects with CD4≥500 cells/mm^3	-0.861	0.779	0.276
CD8+ T-cells			
All subjects*	0.187	0.065	0.005
Subjects with CD4≥500 cells/mm^3	0.279	0.149	0.053
CD4/CD8 ratio			
All subjects*	-0.374	0.094	<0.001
Subjects with CD4≥500 cells/mm^3	-0.723	0.262	0.009

Because of collinearity, we fitted one model to calculate the coefficients of CD4+ and CD8+ T-cells, and a different model for the CD4/CD8 ratio.

Variables CD4+ and CD8+ T-cells, and the CD4/CD8 ratio were log transformed.

Coefficients are adjusted by age, gender, nadir CD4+ T cell count and duration of viral suppression.

To interprete the logarithmically transformed coefficients, we applied the following formula: *Beta*log(1.10)*, resulting in the % of change in the odds of the outcome predicted by each 10% increase in the independent variable.

Table 4. Conditional logistic regression analysis: predicted morbidity and mortality by the CD4+ and CD8+ T-cell counts and the CD4/CD8 ratio in the Madrid cohort and SOCA cohort mortality nested studies.

	Beta	Std. error	P value
Madrid cohort (N=66)			
(all subjects CD4≥500 cells/mm^3)			
CD4+ T-cells			
Unadjusted	-1.86	2.85	0.514
Adjusted by ART duration	-0.66	3.76	0.859
CD8+ T-cells			
Unadjusted	2.80	1.12	0.013
Adjusted by ART duration	2.29	1.16	0.048
CD4/CD8 ratio			
Unadjusted	-6.23	2.48	0.012
Adjusted by ART duration	-5.08	2.53	0.045
SOCA cohort (N=192)			
CD4+ T-cells			
All subjects	-1.52	0.58	0.009
Subjects with CD4≥500 cells/mm^3*	-4.09	6.43	0.525
CD8+ T-cells			
All subjects*	0.28	0.33	0.392
Subjects with CD4≥500 cells/mm^3*	2.37	2.05	0.246
CD4/CD8 ratio			
All subjects*	-1.38	0.55	0.012
Subjects with CD4≥500 cells/mm^3*	-5.04	3.88	0.194

*N=47

Because of collinearity, we fitted one model to calculate the coefficients of CD4+ and CD8+ T-cells, and a different model for the CD4/CD8 ratio.

Variables CD4+ and CD8+ T-cells, and the CD4/CD8 ratio were log transformed.

Coefficients are adjusted by age, gender, nadir CD4+ T cell count and duration of viral suppression.

To interprete the logarithmically transformed coefficients, we applied the following formula: *Beta*log(1.10)*, resulting in the % of change in the odds of the outcome predicted by each 10% increase in the independent variable.

FIGURES

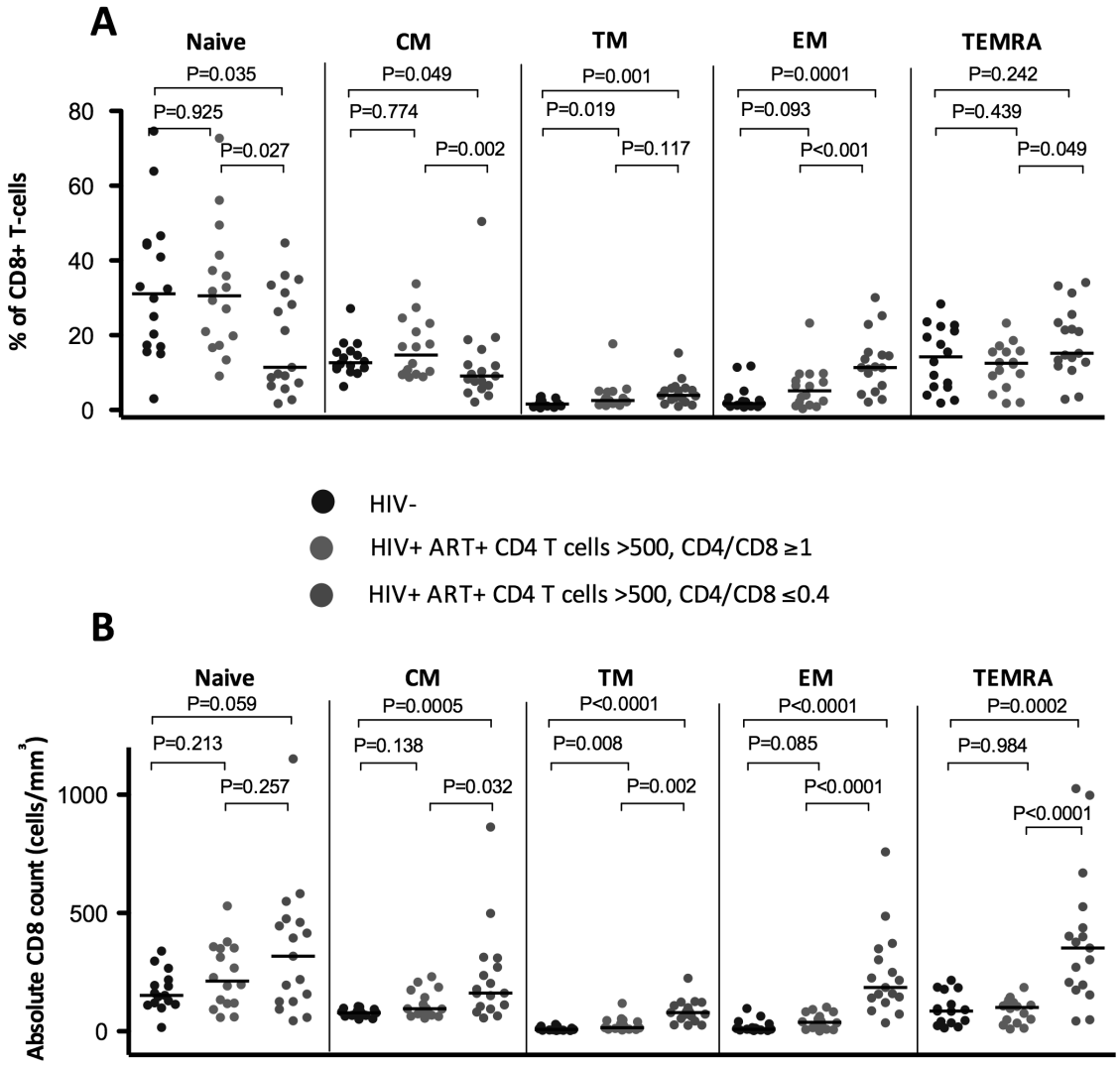


Figure 1. Percentages (A) and absolute counts (B) of CD8+ T-cell maturation subsets among HIV-/CMV+ individuals (blue) and ART-suppressed HIV-infected patients with CD4 counts >500 cells/mm^3 stratified by a normal (4th quartile, ≥1, in green) or low (1st quartile, ≤0.4, in red) CD4/CD8 ratio. HIV-infected individuals with low CD4/CD8 ratio had lower percentages of T_N, T_{CM}, and T_{TR} CD8+ cells, higher T_{EM} and T_{EMRA} **(A)**, and higher absolute counts **(B)** of all subsets compared to those with higher CD4/CD8 ratio and with healthy controls.

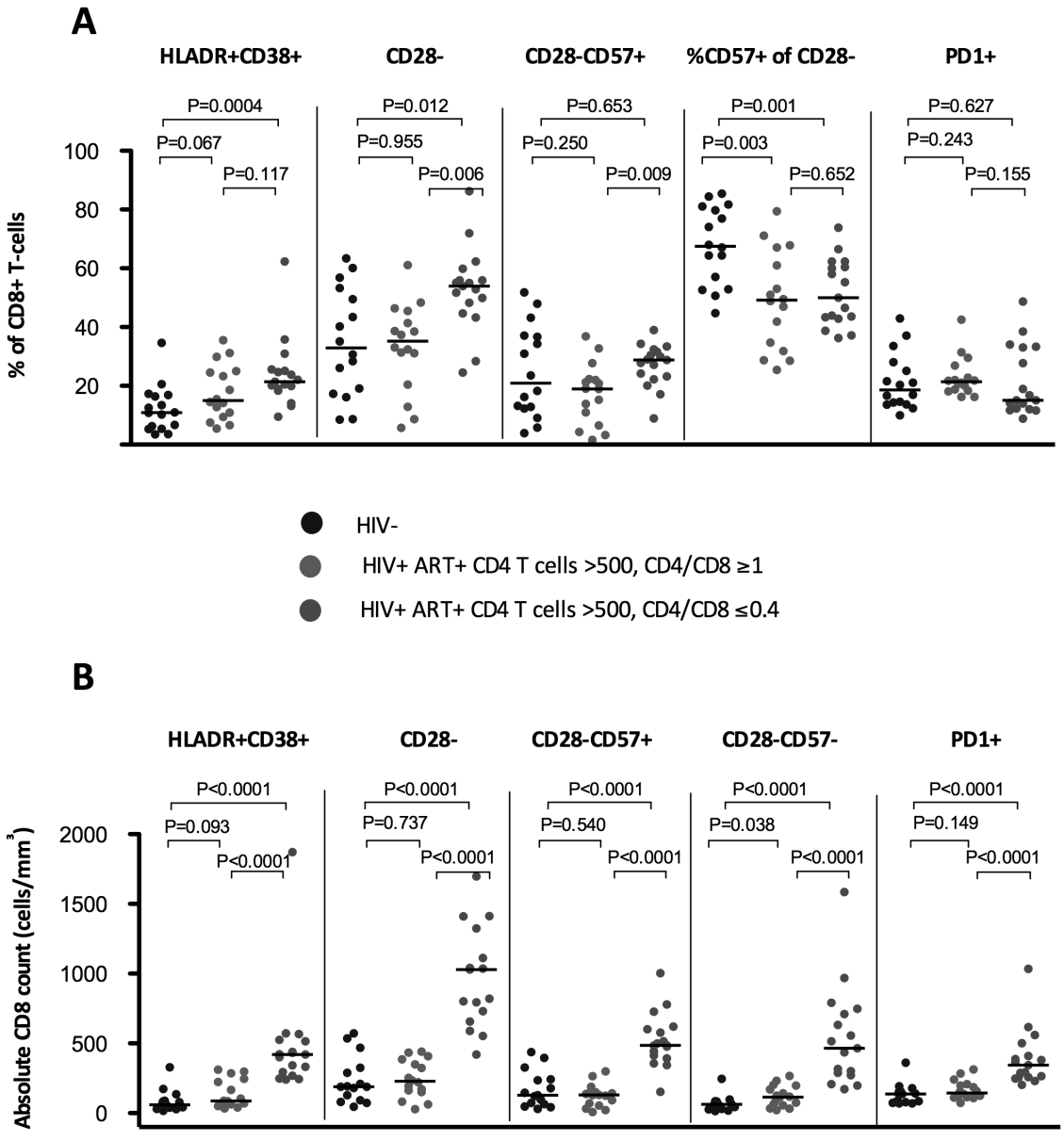


Figure 2. Percentages (A) and absolute counts (B) of CD8+ activation phenotypes among HIV-/CMV+ individuals (blue) and ART-suppressed HIV-infected patients with CD4 counts >500 cells/mm^3 stratified by a normal (4th quartile, ≥1, in green) or low (1st quartile, ≤0.4, in red) CD4/CD8 ratio. Subjects with low CD4/CD8 ratio showed higher percentages **(A)** and absolute counts **(B)** of HLADR+, CD28- and CD28-CD57+, and higher absolute counts of PD1+ cells **(B)**. There were no differences in HIV-infected individuals in the proportion of CD28-CD8+ T-cells expressing CD57, being significantly lower in both groups compared to HIV-/CMV+ controls.

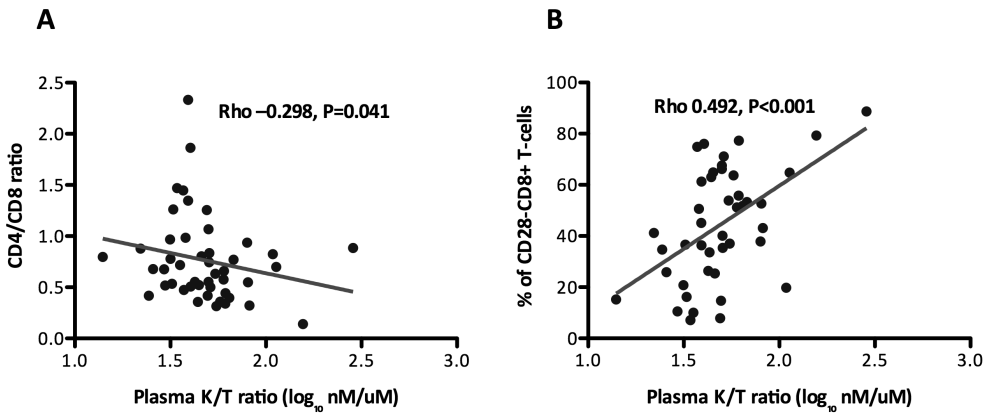


Figure 3. Figure 3. Association between the CD4/CD8 ratio (A) and the % of CD28-CD8+ T-cells (B) with indoleamine 2,3-dioxygenase-1 (IDO-1) activity (kinurenine/tryptophan ratio) among participants in the SOCA cohort with 500 CD4+ T-cells/mm^3. The KT ratio significantly correlated with the CD4/CD8 ratio and the % of CD28+CD8+ T-cells. The between the CD4/CD8 ratio and the KT ratio was confirmed in a linear regression analysis adjusted by age, gender and cumulative ART exposure (Beta=-0.72, P=0.009). The red line represents a linear prediction.

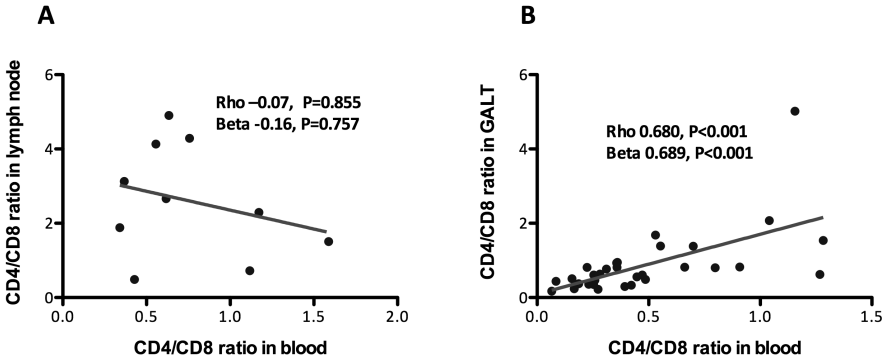


Figure 4. Association between the CD4/CD8 ratio in blood and in lymph nodes (A) or in GALT (B). While no association with the CD4/CD8 ratio in lymph nodes was detected **(A**), it correlated positively with the ratio in GALT **(B)**. The red line represents a linear prediction.

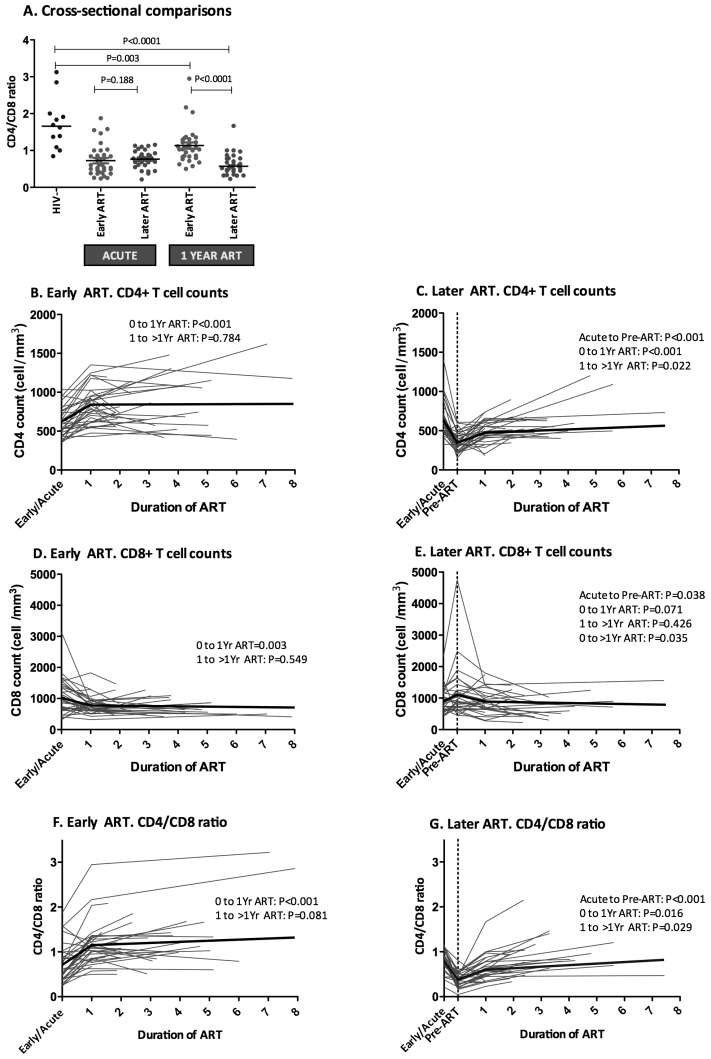
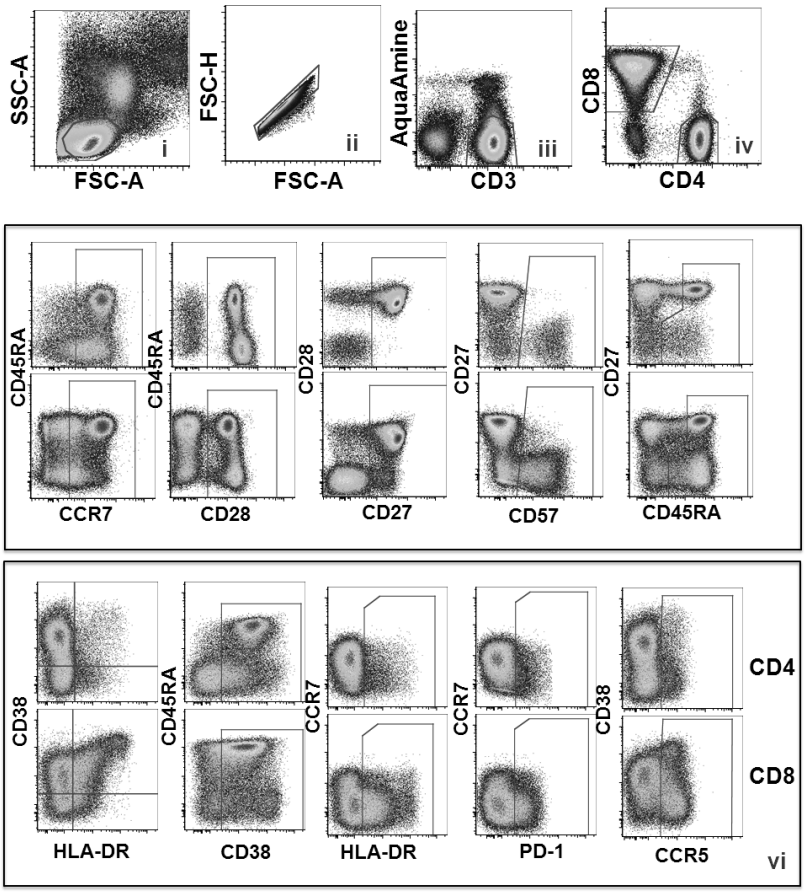


Figure 5. Impact of early or later ART initiation in peripheral CD4+ T-cell counts, CD8+ T-cell counts and CD4/CD8 ratio (A-G) in the OPTIONS cohort. The CD4/CD8 ratio was compared between HIV-uninfected individuals (blue) and HIV-infected individuals initiating ART "early," ≤6 months of infection (green), or "later," ≥2 years after initial infection (red), at acute HIV diagnosis and after 1 year of ART. Median CD4/CD8 ratio was significantly higher after one year in early ART initiators compared to later initiators. **(A)**. Early ART initators experienced higher CD4+ T-cell increase (**B**) than later initiators (**C**) after one year of ART (221 cells/mm^3 vs. 130, respectively, P<0.001). No differences were observed in CD8+ T-cell counts between early (**D**) and later ART initiators (**E**) after one year of ART (-212 cells/mm^3 vs. -114, respectively, P=0.098) but CD8+ T-cells were significantly different between groups beyond one year of ART (-309 cells/mm^3 vs. -114, respectively, P=0.014). Changes in the CD4/CD8 ratio among recently HIV-infected individuals initiating ART early (**F**) and later (**G**) were also assessed over time. Early ART initiators experienced a higher increase at one year of ART than later initiators (+0.43 vs. +0.25, P<0.001). Individual participant trajectories shown with red lines, estimated mean values over time from linear mixed models adjusted by age, sex, baseline CD4+ T-cells shown in thick black lines.



Supplemental Figure 1. Percentages (A) and absolute counts (B) of CD4+ T-cell maturation subsets among HIV-/CMV+ individuals (blue) and ART-suppressed HIV-infected patients with CD4 counts >500 cells/mm^3 stratified by a normal (4th quartile, ≥1, in green) or low (1st quartile, ≤0.4, in red) CD4/CD8 ratio. Individuals with low CD4/CD8 ratio had decreased frequencies of CD4+ T_{TR} and decreased absolute counts of T_N, T_{CM}, and T_{TR} CD4+ T-cells compared to those HIV-infected patients with normal CD4/CD8 ratio and with healthy controls.

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ORIGINAL RESEARCH

The CD4:CD8 ratio is associated with markers of age-associated disease in virally suppressed HIV-infected patients with immunological recovery

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Objectives

Inversion of the CD4:CD8 ratio (< 1) has been identified as a hallmark of inmmunosenescence and an independent predictor of mortality in the general population. We aimed to assess the association between the CD4:CD8 ratio and markers of age-associated disease in treated HIV-infected patients with good immunovirological response.

Methods

A cross-sectional analysis was conducted in 132 HIV-infected adults on antiretroviral therapy (ART), with plasma HIV RNA < 50 HIV-1 RNA copies/mL for at least 1 year, CD4 count > 350 cells/μL and age < 65 years. We analysed the associations between the CD4:CD8 ratio and subclinical atherosclerosis [assessed using carotid intima-media thickness (IMT)], arterial stiffness [assessed using the augmentation index (AIx)], the estimated glomerular filtration rate (eGFR), muscle wasting and sarcopenia [assessed using appendicular lean mass/height^2 (ALM) measured by dual-energy X-ray absorptiometry (DEXA)].

Results

CD4:CD8 ratio inversion was associated with higher IMT, lower eGFR and lower ALM (all values $P < 0.05$), but not with AIx. In multivariate analyses adjusted for age, sex, hypertriglyceridaemia, tobacco use and cumulative ART exposure, inversion of the CD4:CD8 ratio was independently associated with higher IMT [odds ratio (OR) 2.9; 95% confidence interval (CI) 1.2–7.1], arterial stiffness (OR 4.8; 95% CI 1.0–23.5) and lower eGFR (OR 5.2; 95% CI 1.0–64.4), but not sarcopenia (OR 0.7; 95% CI 0.2–2.7). These associations persisted when models were applied to subjects with nadir CD4 counts > 200 cells/μL and those with CD4 counts > 500 cells/μL.

Conclusions

The CD4:CD8 ratio in treated HIV-infected subjects with good immunovirological response is independently associated with markers of age-associated disease. Hence, it might be a clinically useful predictor of non-AIDS-defining conditions.

Keywords: carotid intima-media thickness, CD4:CD8 ratio, HIV, premature aging, sarcopenia, vascular stiffness

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Introduction

Antiretroviral therapy (ART) is among the greatest successes of modern medicine, having rapidly changed the prognosis of HIV-infected individuals from years to decades of

survival. However, ART has failed in its attempt to completely restore normal health to HIV-infected subjects. Although the reasons remain poorly understood, subjects on successful ART still present increased morbidity and mortality relative to uninfected individuals [1–3]. This shortening of the expected life span has recently been associated with increased risk of so-called 'non-AIDS-related' complications, which include cardiovascular disease, renal impairment, liver disease, neurocognitive disorders, non-AIDS-defining cancers, osteoporosis, muscle wasting and frailty. All these complications are generally associated with aging, and concern has been increasing regarding the possibility that persons living with HIV suffer from an 'accelerated aging' syndrome [4]. Most of these noninfectious conditions have been related to the ongoing immune activation and low-level systemic inflammation that occur in chronic HIV infection despite effective ART [4–7]. Although several studies have described immunovirological factors that predict non-AIDS-related end-organ disease risk, such as immune activation, most of these biomarkers are not available in routine clinical practie.

Interestingly, HIV-infected subjects exhibit various changes in the adaptive immune system that are shared by elderly people. Thus, different terms such as 'inflammaging' and immunosenescence have been proposed to allude to this immune phenotype that characterizes both HIV-infected and elderly persons [4,8]. Outside HIV infection, inversion of the CD4:CD8 ratio (< 1), also termed the immune risk profile, is considered a surrogate marker of immunosenescence and independently predicts all-cause mortality [9–12]. Notably, most ART-naïve patients show a low CD4:CD8 T-cell ratio before starting ART, which progressively increases as CD4 counts rise after ART initiation. However, an appreciable number of individuals on long-term treatment still show a low ratio despite CD4 count normalization. In the light of what is known about the CD4:CD8 ratio in the general population, a possible explanation for this finding is the presence of increased immune activation and immunosenescence in these subjects; however, this explanation remains speculative [4]. We have recently reported that the CD4:CD8 ratio is an independent predictor of immune activation in virally suppressed HIV-infected individuals, correlating also with immunosenescence [13]. In a similar analysis in vertically HIV-infected children and adolescents on ART, we found that a low CD4:CD8 ratio was associated with increased levels of CD8 T cells expressing an activation/exhaustion phenotype, and a low naïve : memory T-cell ratio, a feature that also characterizes immunosenescence [14]. Thus, inversion of the CD4:CD8 ratio may identify HIV-infected patients with ongoing immunosenescence and, as a consequence, at higher risk of age-associated diseases [12]. Hence, we hypothesized that an inverted CD4:CD8 ratio despite successful ART may be a marker of non-AIDS-related complications. In this context, we aimed to evaluate whether the CD4:CD8 ratio, which can be obtained in most clinical settings and is routinely measured in HIV-infected patients, was associated in treated HIV-infected patients with markers of subclinical atherosclerosis, arterial stiffness, incipient renal impairment or muscle wasting.

Methods

Study design, participants, setting and eligibility

We conducted an observational, cross-sectional study of 132 consecutive HIV-infected patients who attended a university-based HIV clinic in Madrid between February and October 2011. Subjects were recruited if they were HIV infected and on stable triple ART, defined as continuous treatment with three antiretroviral drugs including either a nonnucleoside reverse transcriptase inhibitor or a protease inhibitor, had had undetectable plasma HIV RNA levels for at least 1 year, and showed CD4 counts ≥ 350 cells/uL at inclusion in the study. As we aimed to analyse variables associated with subclinical aging, exclusion criteria included age > 65 years and the presence of cardiovascular disease (previous stroke, myocardial infarction or intermittent claudication) or chronic kidney disease. The study conformed to the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines and was approved by the local Ethics Committee. All patients gave their written informed consent to participate in the study.

Clinical and laboratory measurements

Medical records were carefully reviewed and all subjects underwent a physical examination. Information on gender, age, body mass index, smoking status, family history of cardiovascular disease and treatment with antiretroviral drugs was recorded. The presence of arterial hypertension, hypercholesterolaemia and hypertriglyceridaemia was defined according to the Adult Treatment Panel III criteria [15]. All patients underwent dual-energy X-ray absorptiometry (DEXA) total body composition measurements, and appendicular lean mass (kg)/height^2 (m^2) (ALM) was used to assess the extent of muscle wasting. We defined sarcopenia as ALM < 2 standard deviations below the mean for young healthy adults (cut-offs were < 7.26 kg/m^2 for men and < 5.45 kg/m^2 for women) [16,17]. A sample of fasting venous blood was obtained to determine concentrations of glucose, high-sensitivity C-reactive protein (CRP), interleukin-6, creatinine, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides using

standard enzymatic methods. Low-densitylipoprotein (LDL) cholesterol concentrations were calculated using the Friedewald equation [18].

Plasma viral load was measured using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Branchburg, NJ). CD4 and CD8 T-cell counts were determined by flow cytometry (Beckman-Coulter, Münster, Germany). Plasma levels of CRP were measured using nephelometry (Siemens Healthcare Diagnostics, Deerfield, IL) and interleukin-6 using chemoluminiscence (Siemens Healthcare Diagnostics). The minimum detection limits of the enzyme-linked immunosorbent assays (ELISAs) for CRP and interleukin-6 were 0.011 mg/L and 1.9 pg/mL, respectively. Typical coefficients of variation for these determinations were < 5%.

Renal function assessment

We used the recently developed Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula to calculate the estimated glomerular filtration rate (eGFR), which has proved to be more accurate than the routinely used Modification of Diet in Renal Disease (MDRD) formula, especially in patients with normal kidney function [19].

Measurement of subclinical atherosclerosis

The degree of subclinical atherosclerosis was evaluated by means of the carotid intima-media thickness (IMT), which has been demonstrated to be an important predictor of cardiovascular events in both the general population [20] and HIV-infected individuals [21], following standard procedures previously described [22]. Briefly, IMT was measured using high-resolution ultrasound (HD7 model; Philips Medical Systems, Eindhoven, The Netherlands) at the common carotid artery (1 cm proximal to the bifurcation) and interpreted using the Mannheim criteria [23]. Measurements were performed by two trained technicians who had previously participated in a pilot study (repeated and blinded measurements performed in 29 patients). The intraclass correlation coefficient was > 0.90.

Measurement of arterial stiffness

The augmentation index (AIx) was used as a surrogate marker of arterial stiffness, which correlates with cardiovascular risk [24]. AIx was measued by pulse wave tonometry, following the recommendations of the European Society of Cardiology [25]. Subjects were examined after resting for at least 5 min in a supine position at a room temperature of 22 ± 1°C. No cigarette smoking or caffeine ingestion was allowed 2 h prior to the examination. Arterial stiffness was assessed noninvasively using the SphygmoCor System (AtCor Medical, Sydney, Australia) and peripheral pressure waveforms were recorded from the right radial artery using applanation tonometry for pulse wave analysis. After 10 sequential waveforms had been acquired, a validated generalized transfer function was used to generate the corresponding central aortic pressure waveform, from which AIx was obtained, which was calculated as the ratio between augmentation pressure and pulse pressure. Because AIx is influenced by heart rate, an index normalized for a heart rate of 75 bpm (AIx@75) was calculated using a general transformation function previously described [26]. Higher values of AIx@75 indicate early return of the reflected wave or increased wave reflection from the periphery as a result of increased arterial stiffness. Subjects were diagnosed with arterial stiffness if AIx@75 was below 2 standard deviations from the reference values in a sex- and age-matched reference population.

Statistical analysis

Qualitative variables were summarized as a frequency distribution and normally distributed quantitative variables as mean ± standard deviation. Continuous nonnormally distributed variables were summarized as median and interquartile range (IQR). Means for variables with a normal distribution were compared using the *t*-test. Nonparametric variables were assessed using the Mann–Whitney test. Given the nonnormal distribution of some of the variables, the Spearman correlation coefficient was used to analyse the correlation between continuous variables.

Independent associations between the CD4:CD8 ratio [as a binary variable: an inverted CD4:CD8 ratio (< 1) *vs.* a normal CD4:CD8 ratio (≥ 1)] and binary outcomes of subclinical aging (IMT > or ≤ p50, presence or absence of arterial stiffness as described above, eGFR > or ≤ p50, and presence or absence of sarcopenia as described above) were evaluated in a multivariate analysis. The modelling strategy involved explanatory logistic regression analysis. Variables with an imbalance between the study groups were introduced into the model, as well as those variables that could exert confounding on the association between the CD4:CD8 ratio and the dependent variables. The variables nadir CD4 and CD8 T-cell counts were excluded from the models because of collinearity. Hence, the maximum model was adjusted by age, sex, hypertriglyceridaemia, tobacco use and cumulative ART exposure. Cumulative tenofovir exposure was also included in the model for renal impairment. As arterial stiffness was defined according to sex and age reference values and sarcopenia according to sex, these demographic variables were not included in the multivariate model for each of these two dependent variables. A backward strategy was used, considering a significance

level of 0.05 to eliminate variables. The overall model was reapplied including only the confounding factors that, after their elimination from the model, produced a ≤ 10% change in the estimate of the coefficient of the variable of principal exposure (inverted CD4:CD8 ratio). The magnitude of association was evaluated using the odds ratio (OR) and 95% confidence interval (CI). In order to assess whether the CD4:CD8 ratio may provide additional information to CD4 nadir and CD4 counts, we applied the multivariate models to the subgroups of patients with low nadir CD4 counts (< 200 cells/μL) and high CD4 counts (> 500 cells/ μL).

The null hypothesis was rejected with a type I error < 0.05 ($\alpha < 0.05$). Statistical analyses were performed using STATA version 12.0 (StataCorp LP, College Station, TX), and figures were generated using GRAPHPAD PRISM 5.00 (GraphPad Software, San Diego, CA).

Results

Table 1 summarizes the main characteristics of the 132 subjects included in the study. The study sample was representative of a middle-aged (47 ± 7 years) HIV-infected population on long-term triple ART (median exposure 7.5 years) and with good immunovirological status (the median CD4 count was 591 cells/mL and 100% of patients had undetectable viral load). Most patients (75%) displayed a low 10-year cardiovascular risk (< 10%), estimated using the Framingham risk score.

All variables assessing subclinical aging – IMT and arterial stiffness for cardiovascular aging, eGFR for renal impairment and ALM as a marker of muscle wasting and sarcopenia – are summarized in Table 1.

Table 1 General characteristics of the study population

Variable (n = 132)	Total
Demographic variables	
Age (years)*	47 (7)
Female [n (%)]	22 (16)
Immunovirological variables	
Viral load < 50 copies/mL [n (%)]	132 (100)
On antiretroviral therapy [n (%)]	132 (100)
Duration of ART (years)	7.5 (3.6–11.8)
Time since HIV diagnosis (years)	11 (7–16)
Nadir CD4 count (cells/μL)	260 (150–381)
CD4 T-cell count (cells/μL)	591 (482–751)
CD8 T-cell count (cells/μL)	813 (607–1078)
CD4:CD8 ratio	0.77 (0.54–1.01)
Cardiovascular risk factors	
Tobacco use (%)	44.4
Hypertension (%)	17.4
Diabetes (%)	11.4
BMI (kg/m^2)	23.7 (22.2–25.7)
Hypercholesterolaemia (%)	48.9
Hypertriglyceridaemia (%)	45.8
Statins (%)	7.6
Variables of age-associated disease	
IMT (mm)*	0.57 (0.11)
Plaque (%)	3.3
Augmentation index (m/s^2)	8.0 (3.5–13.0)
Increased arterial stiffness (%)	21.7
eGFR (mL/min/1.73 m^2)*	93.6 (8.7)
Appendicular lean mass/height (kg/m^2)*	7.1 (1.8)
Sarcopenia (%)	23.8

All values are expressed as median (interquartile range), unless otherwise stated.
ART, antiretroviral therapy; BMI, body mass index; IMT, carotid intima-media thickness; eGFR, estimated glomerular filtration rate.
*Expressed as mean (standard deviation).

Differences in demographic and immunovirological variables, cardiovascular risk factors and inflammation biomarkers according to the CD4:CD8 ratio

Table 2 summarizes the characteristics of patients according to the presence of an inverted (< 1) or normal (≥ 1) CD4:CD8 ratio. A total of 98 patients (74.2%) exhibited inversion of the CD4:CD8 ratio. These individuals were older and were more frequently male, and showed a lower median CD4 count nadir CD4 count nadir compared with patients with a normal CD4:CD8 ratio (all values $P < 0.05$). No significant differences were found between the two groups in the frequency of cardiovascular risk factors, with the exception of a higher frequency of hypertriglyceridaemia ($P < 0.001$) in subjects with a low CD4:CD8 ratio. Levels of inflammatory markers (CRP and interleukin-6) were similar between the two groups.

Associations between subclinical aging and the CD4:CD8 ratio

Next, we explored the linear and categorical associations between the CD4:CD8 ratio and variables associated with subclinical aging, reflecting carotid atherosclerosis (IMT), arterial stiffness (AIx), renal impairment (eGFR) and muscle wasting or sarcopenia (ALM). First, we analysed the linear correlations between the CD4:CD8 ratio and IMT, eGFR and ALM (Fig. 1). The CD4:CD8 ratio was inversely correlated with IMT ($r = -0.192$; $P = 0.037$) (Fig. 1a), but not with AIx ($r = -0.015$; $P = 0.875$) (Fig. 1b), and positively correlated with eGFR ($r = 0.215$; $P = 0.013$) (Fig. 1c). No linear correlation with ALM was detected ($r = -0.169$; $P = 0.134$) (Fig. 1d). We then performed categorical comparisons according to the presence of a normal or an inverted CD4:CD8 ratio. Subjects with inversion of the CD4:CD8 ratio showed increased IMT (Fig. 2a), lower eGFR (Fig. 2c) and lower ALM (Fig. 2c) (all values $P < 0.05$). No statistically significant differences were found in AIx values between subjects with normal and inverted CD4:CD8 ratios. Although patients with inversion of the CD4:CD8 ratio exhibited a higher

Table 2 Differences between individuals with normal and inverted CD4:CD8 ratios

	Inverted CD4:CD8 ratio ($n = 98$)	Normal CD4:CD8 ratio ($n = 34$)	*P*
Demographic variables			
Male/female [*n* (%)]	90 (82)/8 (36)	20 (18)/14 (64)	< 0.001
Age (years)*	48.1 (7.0)	45.2 (7.2)	0.043
Immunovirological variables			
Time to HIV diagnosis (years)	11 (7–17)	11 (8–14)	0.504
Cumulative ART exposure (years)	7.3 (2.8–12.0)	8.8 (4.3–11.8)	0.789
CD4 count nadir (cells/μL)	210 (117–294)	242 (134–318)	0.001
CD4 T-cell count (cells/μL)	541 (457–662)	776 (600–986)	< 0.001
CD8 T-cell count (cells/μL)	935 (699–1127)	605 (486–742)	< 0.001
CD4:CD8 ratio	0.64 (0.46–0.82)	1.21 (1.09–1.42)	< 0.001
Cardiovascular risk factors			
Smoking (%)	43.6	46.92	0.749
Hypertension (%)	16.3	20.6	0.572
Diabetes (%)	12.2	8.8	0.426
BMI (kg/m^2)	24.0 ± 2.8	23.4 ± 2.6	0.295
Hypercholesterolaemia (%)	49.5	47.1	0.808
Hypertriglyceridaemia (%)	51.5	29.4	0.026
Current use of statins (%)	8.2	5.9	0.334
Biomarkers of inflammation			
C-reactive protein (mg/L)	0.16 (0.07–0.42)	0.16 (0.06–0.47)	0.740
Interleukin-6 (pg/mL)	1.9 (1.9–2.2)	1.9 (1.9–2.3)	0.814

All percentages are column percentages, except for sex, which is expressed as row percentages.
All values are expressed as median (interquartile range), unless otherwise stated.
ART, antiretroviral therapy; BMI, body mass index.
*Expressed as mean (standard deviation).

frequency of arterial stiffness, the difference was not statistically significant (11% *vs.* 25%, respectively; $P = 0.122$).

Multivariate analysis

A series of logistic regression models were explored to analyse the association of the CD4:CD8 ratio with variables reflecting subclinical aging (IMT, arterial stiffness, eGFR and sarcopenia), which were used as dependent variables in consecutive multivariate models, adjusting by age, sex, hypertriglyceridaemia, tobacco use and cumulative ART exposure (and specifically tenofovir exposure in the model for renal impairment). In the multivariate analyses, no associations with sarcopenia were observed, but subjects with inversion of the CD4:CD8 ratio showed a significant threefold increased risk of higher IMT and a significant fivefold increased risk of arterial stiffness and lower eGFR (Table 3). Overall, these independent associations and markers of age-associated disease persisted when multivariate models were applied to subjects with nadir CD4 > 200 cells/μL, CD4 count > 500 cells/μL or both.

Discussion

In this study in virally suppressed, HIV-infected subjects, we explored the associations of the CD4:CD8 ratio with markers of vascular disease, kidney disease and muscle wasting. We found that the CD4:CD8 ratio was negatively correlated with IMT, and positively correlated with eGFR (both values, $P < 0.05$). In addition, subjects with inversion of the CD4:CD8 ratio more frequently showed higher IMT, lower eGFR and lower muscle mass (all values, $P < 0.05$). No significant associations were detected with arterial stiffness. In multivariate analyses, inversion of the CD4:CD8 ratio was independently associated with higher IMT, arterial stiffness and lower eGFR. Importantly, these associations remained statistically significant when the sample was censored by low nadir CD4 count (< 200 cells/μL) and low CD4 T-cell count (< 500 cells/μL), suggesting that the CD4:CD8 ratio provides additional information to that provided by nadir CD4 and CD4 T-cell counts as surrogate markers of age-associated disease [27,28]. We also observed that men and older patients more frequently showed inversion of the CD4:CD8 ratio, a finding that is consistent with those of studies in the general population [29,30].

In recent years, consistent data have led to the widespread assumption that HIV infection leads to an increased risk of 'non-AIDS-related' events. Although the underlying mechanisms are not fully understood, a whole body of research suggests that these adverse outcomes are linked to a remodelling of the immune system. Persistent inflammation and immune activation are widely accepted as the major driving factors of this immune senescence and exhaustion that ultimately lead to disease progression and

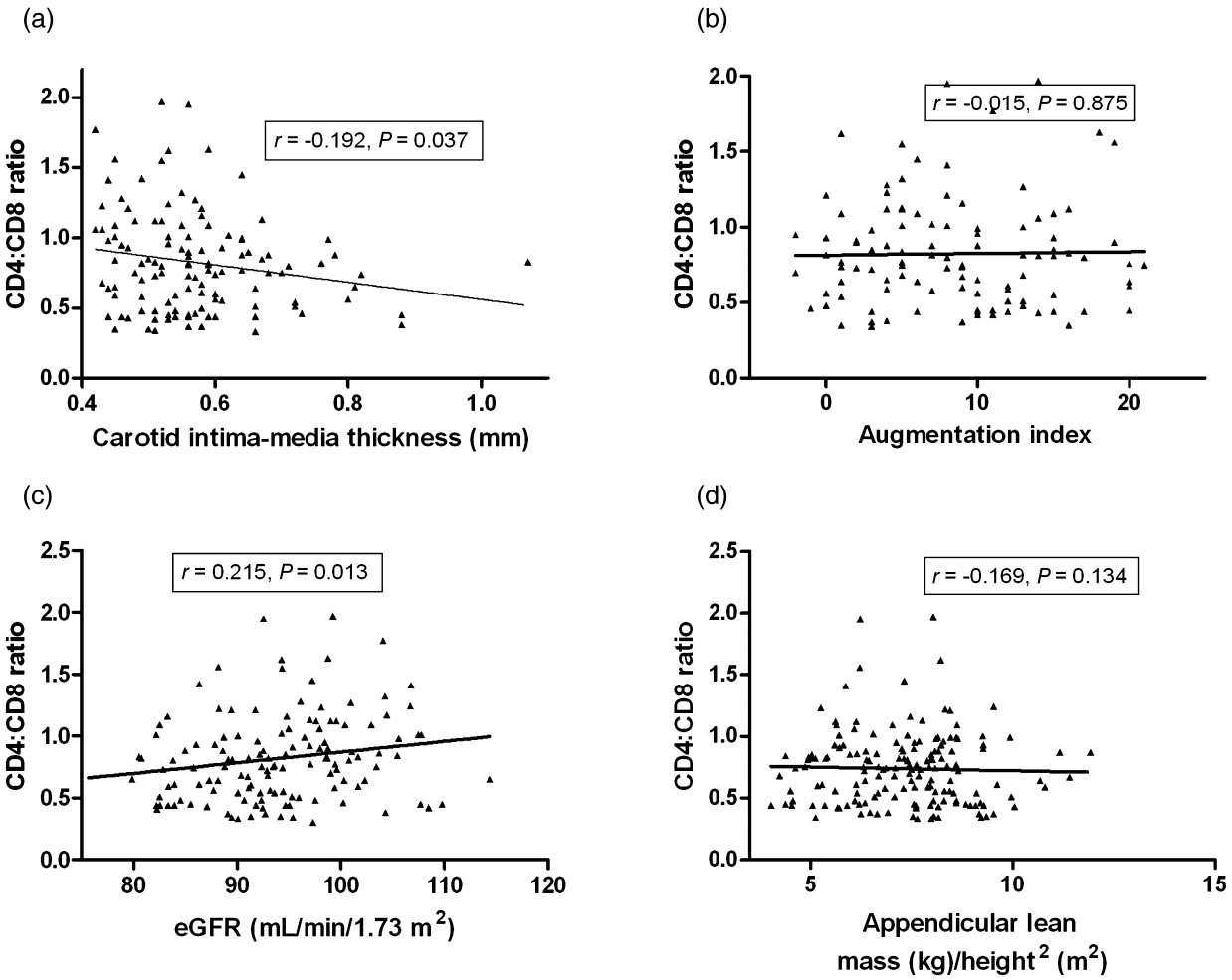

Fig. 1 Linear correlations between the CD4:CD8 ratio and different markers of age-associated disease. The CD4:CD8 ratio was inversely correlated with carotid intima-media thickness (IMT) ($r = -0.192$; $P = 0.037$) (a), but not with the augmentation index ($r = -0.015$; $P = 0.875$) (b), and positively correlated with the estimated glomerular filtration rate (eGFR) ($r = 0.215$; $P = 0.013$) (c). No linear correlation with appendicular lean mass/height^2 (ALM) was detected ($r = -0.169$; $P = 0.134$) (d).

adverse outcomes [31]. Thus, many groups have focused on the study of biomarkers of inflammation and surrogate markers of non-AIDS-related events during treated HIV infection [32]. However, none of these markers has been properly validated and, despite the fact that viral control and immunological restoration no longer pose a clinical challenge, management of HIV-infected patients is still based on optimization of the same surrogate parameters of immunovirological control as were used 30 years ago: viral load and CD4 T-cell count.

Most studies addressing the problem of premature aging in HIV disease have focused on ART-naïve individuals, and have shown that naïve HIV-infected subjects share diverse immunological changes with elderly people. These similarities include a low naïve : memory T-cell ratio, expansion of cytomegalovirus (CMV)-specific CD8 T cells, higher percentages of $CD57^+CD27^-$ ('senescent') and $PD\text{-}1^+$ ('exhausted') T cells, increased CRP and interleukin-6 levels, reduced responses to vaccines, reduced T-cell telomere lengths and a low CD4:CD8 ratio [33–36]. However, the extent to which ART reverses these HIV-induced immune changes is currently the subject of ongoing investigation. Interestingly, most individuals (74%) in our study exhibited an inverted CD4:CD8 ratio despite at least 1 year of ART-mediated plasma viral suppression and CD4 T-cell count recovery. To the best of our knowledge, the clinical significance of this phenomenon in long-term ART-experienced subjects has not yet been investigated. Outside HIV infection, a low CD4:CD8 ratio, also termed the immune risk profile, is considered a

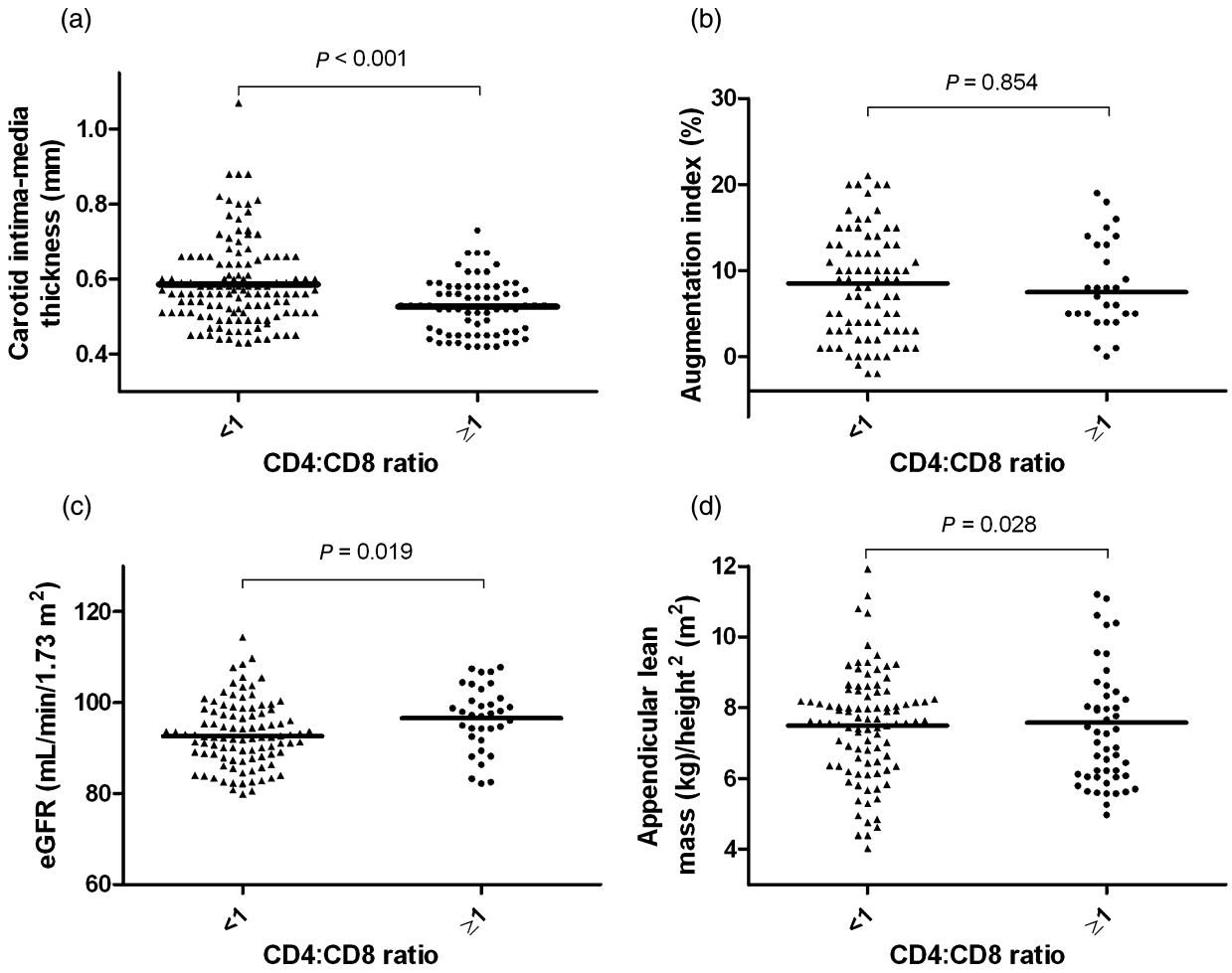

Fig. 2 Categorical associations between the CD4:CD8 ratio and markers of age-associated disease. Subjects with inversion of the CD4:CD8 ratio showed increased carotid intima-media thickness (IMT) (a), lower estimated glomerular filtration rate (eGFR) (b) and lower appendicular lean mass/height^2 (ALM) (c) (all values $P < 0.05$). No statistically significant differences were found in augmentation index (AIx) values between subjects with normal and inverted CD4:CD8 ratios. Although patients with inversion of the CD4:CD8 ratio exhibited a higher frequency of arterial stiffness, the difference was not statistically significant (11% *vs.* 25%, respectively; $P = 0.122$).

surrogate marker of immunosenescence and has been shown to be an independent predictor of all-cause mortality [11,37]. The immune risk profile is characterized by an increase in the number of $CD8^+CD28^-$ cells, which results in a low CD4:CD8 ratio, and is associated with a high number of CMV-specific T cells or CMV seropositivity [9]. Similarly, in HIV infection, persistence of a low CD4:CD8 ratio despite CD4 T-cell count restoration may be explained by oligoclonal expansion of CD8 T cells, reflecting an underlying immunosenescence [9], raising the possibility that the CD4:CD8 ratio may also be a surrogate marker of immunosenescence in this population.

On the basis of this hypothesis, we recently explored the biological significance of the CD4:CD8 ratio in treated HIV-infected patients. In a cross-sectional analysis in 20 subjects with long-standing viral suppression, we observed that the CD4:CD8 ratio was an independent predictor of CD4 T-cell activation [13]. We also addressed the association between the CD4:CD8 ratio and T-cell activation in 37 vertically HIV-infected children and adolescents, finding that the inversion of the CD4:CD8 ratio was a strong independent predictor of higher percentages of CD8 T cells expressing activated ($HLA\text{-}DR^+CD38^+$), senescent ($CD58^+CD27^-$) and activated/exhausted ($HLA\text{-}DR^+PD\text{-}1^+$) phenotypes [14]. In view of the fact that immune activation is considered the major driving factor of this premature aging in HIV infection, these data suggested that inversion of the CD4:CD8 ratio might help to identify ART-treated patients at higher risk of noninfectious conditions. The present study provides new insight into the clinical significance of the CD4:CD8 ratio in

Table 3 Multivariate analyses: independent associations with the CD4:CD8 ratio

	CD4:CD8 ratio < 1 (%) (*n* = 98)	CD4:CD8 ratio ≥ 1 (%) (*n* = 34)	Adjusted OR	95% CI	*P*
IMT (mm) > p50*					
All	50.0	25.8	2.9	1.2–7.1	0.022
CD4 count > 500 cells/μL	53.6	25.6	3.3	1.2–9.0	0.020
Nadir CD4 count > 200 cells/μL	50.0	25.0	3.0	1.1–8.0	0.028
Arterial stiffness†					
All	25.3	11.1	4.8	1.0–23.5	0.048
CD4 count > 500 cells/μL	33.9	12.5	4.8	1.0–23.2	0.048
Nadir CD4 count > 200 cells/μL	28.6	8.3	8.0	1.0–64.4	0.050
eGFR (mL/min/1.73 m^2) ≤ p50†					
All	59.2	26.5	5.2	1.3–20.0	0.017
Nadir CD4 count > 200 cells/μL	65.3	29.0	5.0	1.2–21.4	0.028
CD4 count > 500 cells/μL	64.5	30.0	4.2	0.97–18.5	0.056
Sarcopenia†					
All	23.7	23.8	0.7	0.2–2.7	0.951
Nadir CD4 count > 200 cells/μL	28.3	21.1	0.5	0.1–2.9	0.556
CD4 count > 500 cells/μL	25.7	26.3	0.6	0.2–2.7	0.932

CI, confidence interval; IMT, carotid intima-media thickness; eGFR, estimated glomerular filtration rate; OR, odds ratio.
Maximum multivariate models were adjusted by:
*age, sex, tobacco use, hypertriglyceridaemia and cumulative ART exposure;
†tobacco use, hypertriglyceridaemia and cumulative ART exposure;
‡age, tobacco use, hypertriglyceridaemia and cumulative ART exposure.

treated HIV-infected subjects, as we have demonstrated its value as a variable independently associated with various aspects of the aging process: atherosclerosis, arterial stiffness and renal impairment.

Our study is subject to a number of limitations common to cross-sectional studies. In addition, we used surrogate markers of cardiovascular risk – IMT and arterial stiffness – instead of analysing cardiovascular events, which would have required a prospective study with a large sample size. Although IMT has been demonstrated to be an important predictor of cardiovascular events in the general population [20], and IMT measurement is the test recommended by the American Heart Association for the assessment of the atherosclerotic burden [21], a recent meta-analysis in the general population found no evidence of increased risk of cardiovascular events associated with faster IMT progression [38]; this recent study precludes us from drawing the conclusion that inversion of the CD4:CD8 ratio in treated HIV-infected patients may help to identify subjects at increased cardiovascular risk. Our sample was representative of a middle-aged, low-cardiovascular-risk population according to the Framingham risk score equation. Accordingly, we expected to have a low proportion of subjects with high IMT values (> 1 mm). As IMT correlates with the incidence of cardiovascular events and no threshold effect is observed [20], we considered use of the median IMT as an unbiased way to stratify subjects according to their degree of subclinical atherosclerosis. Similarly, we classified subjects according to their eGFR with a cut-off within the normal range (90–120 mL/min/1.73 m^2). Of note, each 10 mL/min/1.73 m^2 decrease in eGFR has been associated with a 20% increased OR of cardiovascular events in HIV-infected patients [39], and previously we have reported that incipient renal impairment is an independent marker of subclinical atherosclerosis in HIV-infected individuals [22]. Finally, only 22 women were included in this study (17%) and thus our results cannot be extrapolated to women.

All things considered, our data suggest that the CD4:CD8 ratio in HIV-infected subjects on effective ART is independently associated with surrogate markers of age-associated disease. Although the discriminative ability of the CD4:CD8 ratio as a marker of non-AIDS-related events must be confirmed in large prospective studies with clinical endpoints, we believe that the description of a surrogate marker of accelerated aging in successfully treated HIV-infected individuals may have important implications in both clinical and investigational settings. If the CD4:CD8 ratio is further validated as an independent predictor of non-AIDS-related morbidity, normalization of the CD4:CD8 ratio might be considered a clinical target. As the findings of two previous studies from our group also support the conclusion that the CD4:CD8 ratio is independently associated with immune activation [13,14], and immune activation seems to drive non-infectious-disease-associated mortality and morbidity in treated HIV infection [5,40], it may be of great interest to include subjects with a low CD4:CD8 ratio despite successful ART in clinical trials of interventions aiming to reduce chronic inflammation and immune activation. Also, treated individuals with

the lowest CD4:CD8 ratios despite long-term viral suppression might be specially monitored for the prompt detection and early treatment of age-related conditions. It is noteworthy that the CD4:CD8 ratio is usually reported and available in routine clinical practice; thus, its use as a predictor of non-AIDS-related disorders might be easily implemented if more evidence is obtained to support our data.

In conclusion, in this study in treated HIV-infected subjects, a low CD4:CD8 ratio despite at least 1 year of effective ART was an independent marker of conditions associated with age-related disease – carotid atherosclerosis, arterial stiffness and renal impairment. These findings may have implications in both research and clinical settings.

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Diagnosis of subclinical atherosclerosis in HIV-infected patients: higher accuracy of the D:A:D risk equation over Framingham and SCORE algorithms

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Abstract

Aims: While the detection of subclinical atherosclerosis may provide an opportunity for the prevention of cardiovascular disease (CVD), which currently is a leading cause of death in HIV-infected subjects, its diagnosis is a clinical challenge. We aimed to compare the agreement and diagnostic performance of Framingham, SCORE and D:A:D equations for the recognition of subclinical atherosclerosis in HIV patients and to adjust the D:A:D equation using HIV and CVD variables.

Methods and results: Atherosclerosis was evaluated in 203 HIV-infected individuals by measuring the carotid intima-media thickness (IMT). The CVD risk was calculated using the Framingham, SCORE and D:A:D risk equations. Framingham, SCORE and D:A:D equations showed a low agreement with the IMT (Kappa: 0.219, 0.298, 0.244, respectively; $p = 0.743$) and a moderate predictive performance, (area under the curve [AUC] = 0.686, 0.665 and 0.716, respectively; $p = 0.048$), with the D:A:D equation being the most accurate. Atherosclerosis was demonstrated in a significant proportion of subjects with low predicted CVD risk by all three algorithms (16.3%, 17.2%, 17.2%, respectively; $p = 0.743$). In patients with an estimated low CVD risk atherosclerosis was associated with older age ($p = 0.012$) and low CD4 counts ($p = 0.021$). A model was developed to adjust the D:A:D equation; a significant increase in accuracy was obtained when CD4 counts and low-grade albuminuria were included (AUC = 0.772; $p < 0.001$).

Conclusion: The D:A:D equation overperforms Framingham and SCORE in HIV patients. However, all three equations underestimate the presence of subclinical atherosclerosis in this population. The accuracy of the D:A:D equation improves when CD4 counts and low-grade albuminuria are incorporated into the equation.

Keywords

HIV, atherosclerosis, Framingham risk equation, SCORE, D:A:D risk equation, carotid intima-media thickness

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Introduction

The natural history of HIV infection has dramatically changed following the introduction of highly active antiretroviral therapy (HAART) in 1996.[1] As result, the incidence of AIDS-related deaths and opportunistic infections has been markedly reduced in Western countries, and non-AIDS diseases including cardiovascular, hepatic, pulmonary and non-AIDS defining malignancies have become the leading causes of death.[2] As an example, the adjusted hazard ratio for the incidence of coronary events in HIV-infected persons is 1.75,[3] and ischaemic heart disease has proved to be the principal cause of non-HIV-related deaths in HIV persons 55 years of age or older in New York City.[4] Given the fact that the mean age of HIV-infected individuals is progressively increasing,[5] cardiovascular disease (CVD) is likely to gain further importance as a cause of mortality in years to come.

Thus, the prompt detection of CVD at earliest stages is crucial for implementing preventive measures in patients at higher risk. At the moment, the decision about initiation or deferral of treatment to prevent CVD is generally based on the individuals' risk estimation generated from equations derived from the general population.[6] Although the Framingham equation is the most widely used estimation, it was developed in HIV-uninfected subjects from the US,[7] which may explain its inaccuracy when applied to other populations. Indeed, several reports have highlighted that in HIV patients the Framingham equation may underestimate the real CVD risk in subjects with a longer duration of antiretroviral therapy.[8,9] Similar analyses have not been performed yet using the algorithm recommended for European populations, named the Systematic Coronary Risk Evaluation model (SCORE).[10] More recently, the Data Collection on Adverse Effects of Anti-HIV Drugs (D:A:D) group has developed a risk equation from a population of HIV-infected patients, incorporating routinely collected cardiovascular risk parameters and exposure to certain antiretroviral drugs. This new equation provided a better estimation of the risk of CVD than the Framingham equation in HIV-infected persons.[11]

The measurement of the carotid intima-media thickness (IMT) is a precise, reproducible and non-invasive approach to detect subclinical atherosclerosis. This tool has proved to be an important predictor of cardiovascular events.[12–14] It is noteworthy that HIV-infected patients show thicker IMT than the general population.[15,16]

It is currently unknown whether the SCORE equation predicts better subclinical atherosclerosis than Framingham in European HIV-infected patients. On the other hand, the diagnostic performance of the D:A:D equation for the detection of subclinical atherosclerosis has not been investigated yet. The purpose of our study was to compare the diagnostic performances of Framingham, SCORE and D:A:D equations for the detection of subclinical atherosclerosis in a population of HIV-infected subjects and to analyse the effect of including HIV and CVD variables in the D:A:D equation.

Methods

Study design, participants, setting and eligibility

We conducted an observational, cross-sectional study of 203 consecutive HIV-infected patients who attended a university-based HIV clinic in Madrid in years 2009 and 2010. Patients were excluded in the presence of known CVD (previous stroke, myocardial infarction, or intermittent claudication) and/or chronic kidney disease. The local Ethics Committee approved the study and all patients gave their written informed consent to participate.

Clinical and laboratory measurements

Medical records were carefully reviewed, a questionnaire was completed, and physical examination was performed. Information on gender, age, body mass index, smoking status, family history of CVD, and treatment with antiretroviral drugs was recorded. The presence of arterial hypertension, hypercholesterolemia, hypertriglyceridemia, and metabolic syndrome was defined according to the Adult Treatment Panel III criteria.[17] Lipodystrophy was defined as the presence of body fat changes that could be clearly recognized by the patient and confirmed by the doctor, and diagnosed following the lipodystrophy severity grading scale of Lichtenstein et al.[18]

A sample of fasting venous blood was obtained to determine concentrations of glucose, interleukin-6, homocystein, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides using standard enzymatic methods. Low-density lipoprotein (LDL) cholesterol concentrations were calculated using the Friedewald equation.[19]

Plasma viral load was measured using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Inc., Branchburg, NJ), and CD4 lymphocyte count were determined by flow cytometry (Beckman-Coulter, Inc., Münster, Germany). Patients were considered to be immune suppressed when the CD4 count was below than 250 cells/μl. Hepatitis C virus coinfection was diagnosed in the presence of a positive serology and detectable viremia.

Plasma levels of high-sensitivity C-reactive protein were measured using nephelometry (VISTA Systems, Siemens Healthcare Disgnostics, Inc., Deerfield, IL). D-dimers were measured using turbidimetry (Beckman-Coulter, Inc., Münster, Germany) and fibrinogen concentration using a prothrombin time-derived method. Plasma levels of LP-PLA2 were determined using a commercial enzyme immunoassay (PLAC™ Test, diaDexus, Inc., CA, USA).

A urine sample was collected from each participant and urinary albumin and creatinine concentrations were determined by turbidimetry (Olympus Diagnostics AU2700 autoanalyser, Inc., Hamburg, Germany).[18] Albuminuria was calculated using the albumin/creatinine urine ratio (ACR).

CVD risk assessment

The risk of CVD was assessed in all patients using Framingham,[7,17] SCORE calibrated for the Spanish population[10,20] and D:A:D[11] risk equations. Whereas Framingham and SCORE equations estimate the risk of CVD within the next 10 years, the D:A:D equation provides a risk estimation for the next 5 years. Therefore, in order to compare properly the performance of all three equations, D:A:D predictions were projected over 10 years.[11] Subsequently, patients were categorized on the basis of two levels of CVD risk at 10 years: low (<10% for Framingham and D:A:D, <5% for SCORE) and high (≥10% for Framingham and D:A:D, ≥5% for SCORE).

Measurement of IMT

IMT was examined using ultrasonography (HD7 model, Philips Medical Systems, Inc., Eindhoven, The Netherlands) and interpreted using the Mannheim criteria.[21,22] Measurements were made at the common carotid artery (1 cm proximal to the bifurcation). Far wall IMT images were obtained and digitalized for each patient. An IMT detection software was previously calibrated using QLab (Philips Medical Systems, Inc., Eindhoven, the Netherlands).[23] More than 400 measurements were performed for each patient, and the median value was used for the final statistical analyses. A plaque was defined as a thickness >1.5 mm or a focal structure that encroached into the arterial lumen by at least 0.5 mm or at least 50% of the surrounding IMT value. As IMT correlates linearly with the incidence of coronary events,[13] participants were categorized into two groups: those who presented an IMT ≥75th percentile in the study population (0.64 mm) or in whom a plaque was demonstrated were considered at increased risk of CVD and were classified as having subclinical atherosclerosis; the remaining patients were included in the comparison group. Several factors were taken into consideration before IMT was classified as being above the 75th percentile. First, carotid IMT correlates with the incidence of cardiovascular events and no threshold effect is observed.[13] Second, IMT depends largely on age; however, reference values of IMT adjusted for age are lacking in our population. Third, in our study the dispersion in mean age was slight. Finally, the 75th percentile is indicative of increased CVD risk according to the American Society of Echocardiography Carotid Intima-Media Thickness Task Force.[24] Measurements were performed by two trained technicians who had previously participated in a pilot study (repeated and blinded measurements performed in 29 patients). The intraclass correlation coefficient was >0.90.

Statistical analysis

Qualitative variables were reported as a frequency distribution whereas normally distributed quantitative variables were described as mean ± standard deviation. The continuous non-normally distributed variables were reported as median and interquartile ranges (IQRs). Means for variables with a normal distribution were compared using the Student's *t*-test or the analysis of variance (ANOVA). Non-parametric variables were examined using the Kruskal–Wallis test.

The kappa coefficient was used as a measure of the agreement between the presence of subclinical atherosclerosis and the risk calculated by Framingham, SCORE and D:A:D equations. Their diagnostic values for the detection of subclinical atherosclerosis were analysed using the Receiver Operating Characteristic (ROC) curve and calculating the area under curve (AUC) for each of the continuous CVD assessments. Linear associations between IMT and Framingham, SCORE and D:A:D risk equations were evaluated by the non-parametric Spearman's rank correlation.

Logistic regression analysis was used to examine the variables associated with the presence of subclinical atherosclerosis (as a binary variable) in the group of patients with low CVD risk informed by the D:A:D equation. Variables included in the multivariate analyses were those statistically significant in the univariate analysis and/or clinically relevant.

The diagnostic performance of the D:A:D equation was adjusted by calculating the increases in the AUC when a combination of different variables was added to D:A:D equations by logistic regression analysis. The null hypothesis was rejected by a type I error <0.05 ($\alpha < 0.05$). All statistical analyses were performed using the SPSS 15.0 statistical package (SPSS, Inc., IL, USA).

Table 1. Main characteristics of the study population. Differences in the estimation of CVD risk predicted by the D:A:D equation and the observed subclinical atherosclerosis

	All	No Atherosclerosis D:A:D < 10%	Atherosclerosis D:A:D < 10%	Atherosclerosis D:A:D > 10%	*p*-value*
N	203	123	35	22	
Age (years)	46.7 ± 10.5	42.0 ± 7.7	48.4 ± 7.2	61.8 ± 10.1	<0.001
Male, %	85.9	82.1	88.6	95.5	0.363
Cardiovascular risk factors					
BMI (kg/m^2)	23.7 ± 2.7	23.5 ± 2.7	24.4 ± 2.6	23.9 ± 3.2	0.107
Smokers, %	46.0	39.8	37.1	54.5	0.773
Hypertension, %	16.9	10.6	25.7	18.	0.022
Diabetes mellitus, %	9.9	2.4	8.6	40.4	0.094
Hypercholesterolemia, %	39.4	37.4	31.4	50.0	0.516
Hypertriglyceridemia, %	43.2	30.9	51.4	59.1	0.011
Metabolic syndrome, %	11.7	6.9	20.6	25.0	0.019
HIV-related variables					
CD4 count (cells/μl)	517 (379–688)	532 (382–728)	441 (256–693)	502 (405–658)	0.163
CD4 nadir (cells/μl)	270 (164–399)	296 (186–427)	236 (143–386)	177 (61–332)	0.262
CD4 <250 (cells/μl), %	10.4	7.4	22.9	9.1	0.009
Viral load <50 copies/ml, %	73.9	83.7	85.3	77.3	0.051
On antiretroviral therapy, %	88.7	86.3	100	90.9	0.011
Time to HIV diagnosis (years)	10 (4–16)	9.0 (3.0–15.0)	11.0 (6.0–18.0)	13.5 (6.3–19.3)	0.017
Exposure to NRTI (months)	138 (45.5–245.5)	94 (26–194)	186 (72–258)	232.5 (133.5–256.8)	0.004
Exposure to abacavir (months)	0 (0–19)	0 (0–1)	0 (0–32)	0 (0–41.5)	0.377
Exposure to NNRTI (months)	23 (0–54.5)	20 (0–45)	24 (0–83)	29 (7–86)	0.522
Exposure to PI (months)	26 (0–93.5)	0 (0–82)	42 (2–77)	40 (18–150)	0.032
Hepatitis C coinfection, %	25.8	25.2	34.4	19.0	0.306
Biomarkers of vascular damage					
C-reactive protein (mg/l)	0.16 (0.07–0.41)	0.14 (0.06–0.32)	0.12 (0.05–0.48)	0.27 (0.18–0.49)	0.917
Interleukine-6 (pg/ml)	1.9 (1.9–2.8)	1.9 (1.0–2.7)	1.9 (1.9–2.5)	1.0 (1.9–3.6)	0.972
LpPLA-2 (ng/ml)	234.9 ± 75.8	235.4 ± 83.0	240.7 ± 74.3	219.9 ± 58.3	0.918
Fibrinogen (mg/dl)	319.0 ± 81.3	309.8 ± 71.5	320.8 ± 88.6	335.7 ± 119.8	0.777
Homocystein (μmol/l)	22.0 (8.7–12.7)	10.5 (8.2–12.7)	10.7 (9.4–12.2)	11.9 (11.4–17.1)	0.444
D-dimers (ng/ml)	175.0 (81.3–328.3)	175 (87–319)	167 (71–348)	336 (115–548)	0.862
Albumin/creatinine urine (mg/g)	4.1 (2.6–8.9)	3.3 (2.4–5.9)	7.0 (3.6–15.3)*	11.2 (4.7–25.6)	0.001

Results are presented as mean (standard deviation) for variables with parametric distributions, and as median (range) for variables with non-parametric distributions; CVD, cardiovascular disease; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitors; *p-value < 0.05 for comparison between the group 'No atherosclerosis and D:A:D < 10%' and the group 'Atherosclerosis and D:A:D < 10%'.

Results

Table 1 summarizes the main characteristics of the 203 HIV-infected participants included in the study. The population was stratified into three groups according to the estimation of cardiovascular risk using the D:A:D equation and the observed subclinical atherosclerosis. The study population was mainly represented by a middle-aged (46.7 ± 10 years) HIV-positive male group. The most frequent CVD risk factor was smoking (46.0%), followed by hypertriglyceridemia (43.2%), hypercholesterolemia (39.4%), hypertension (16.9%), and diabetes mellitus (9.9%). All subjects with hypertension were on antihypertensive drugs (angiotensin-converting enzyme inhibitors or angiotensin-II receptor blockers). Metabolic syndrome was detected in 11.7% of individuals. Most patients were on HAART (88.7%) and had an undetectable viral load (73.9%). Mean IMT was 0.58 ± 0.13 mm. A plaque was demonstrated in 10 (5.0%) cases and

57 (26.8%) individuals were found to have subclinical atherosclerosis.

Patients with a low risk of CVD according to the D:A:D equation and with atherosclerosis were older, had an increased waist circumference and more frequently showed hypertriglyceridemia, diabetes and metabolic syndrome. Regarding HIV-related variables, these subjects more frequently presented lipodystrophy, immune suppression, a lowest CD4 nadir, a longer duration of HIV infection and more accumulated exposure to non-nucleoside reverse transcriptase inhibitors.

Regarding biomarkers of CVD risk (C-reactive protein, interleukin-6, LP-PLA2, fibrinogen, homocystein, D-dimers, ACR), only ACR increased across the groups of CVD risk with statistical significance.

Figure 1 shows the linear relationship between IMT and Framingham, SCORE and D:A:D risk equations. All-three equations showed a positive correlation with IMT of similar intensity. The estimation of CVD risk according to Framingham, SCORE and D:A:D equations in the study population is summarized in Figure 2. All equations shared a low level of agreement with the presence of atherosclerosis by measurement of the IMT, with no significant differences among them (Kappa: 0.219, 0.298, 0.244, respectively; $p = 0.743$). All models showed a moderate predictive performance, being the D:A:D equation the most accurate (AUC = 0.686, 0.665, 0.716, respectively; $p = 0.048$). Of note, subclinical atherosclerosis was demonstrated in an important proportion of HIV subjects with a low prediction of CVD risk (16.3%, 17.2%, 17.2%, respectively; $p = 0.768$).

The independent predictors of subclinical atherosclerosis were examined adjusting for age, metabolic syndrome, nadir CD4 cell count, time from HIV diagnosis, immune suppression (CD4 counts < 250 cells/μl), and accumulated exposure to nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and/or protease inhibitors.

Logistic regression analysis showed that variables significantly associated with the presence of subclinical atherosclerosis in patients with an estimated low risk of CVD by D:A:D were older age (odds ratio [OR] 2.8; 95% confidence interval [CI] 1.3–6.4; $p = 0.012$] and a low CD4 cell count (<250 cells/μl) (OR 3.7; 95% CI 1.2–10.9; $p = 0.021$).

In order to identify the model with the best predictive performance, different combinations of variables related to HIV and CVD were introduced in the D:A:D equation using a binary logistic regression analysis (Table 2). The best accuracy was obtained when the CD4 counts and the ACR were included in the D:A:D equation, with an increase in the AUC of 8% (AUC = 0.772; $p < 0.001$).

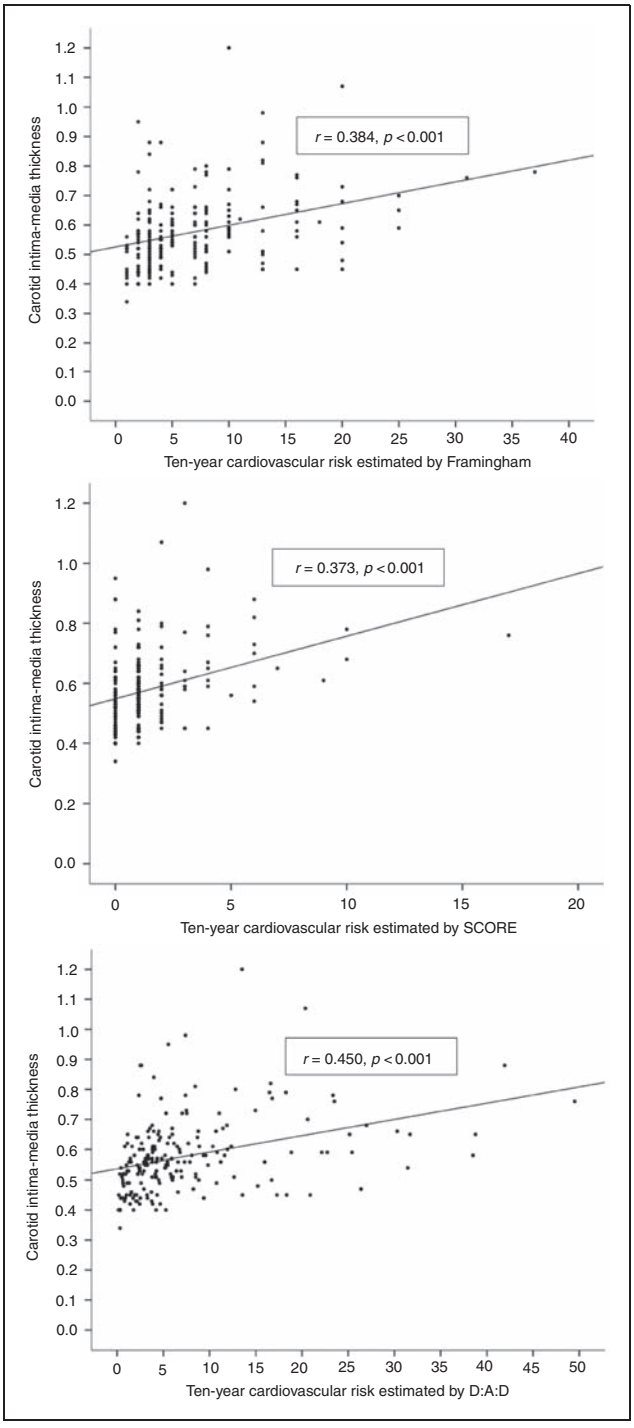

Figure 1. Linear associations between intima-media and Framingham, SCORE and D:A:D risk equations.

Discussion

Our results suggest that the D:A:D equation predicts better than Framingham and SCORE the presence of subclinical atherosclerosis in HIV-infected individuals.

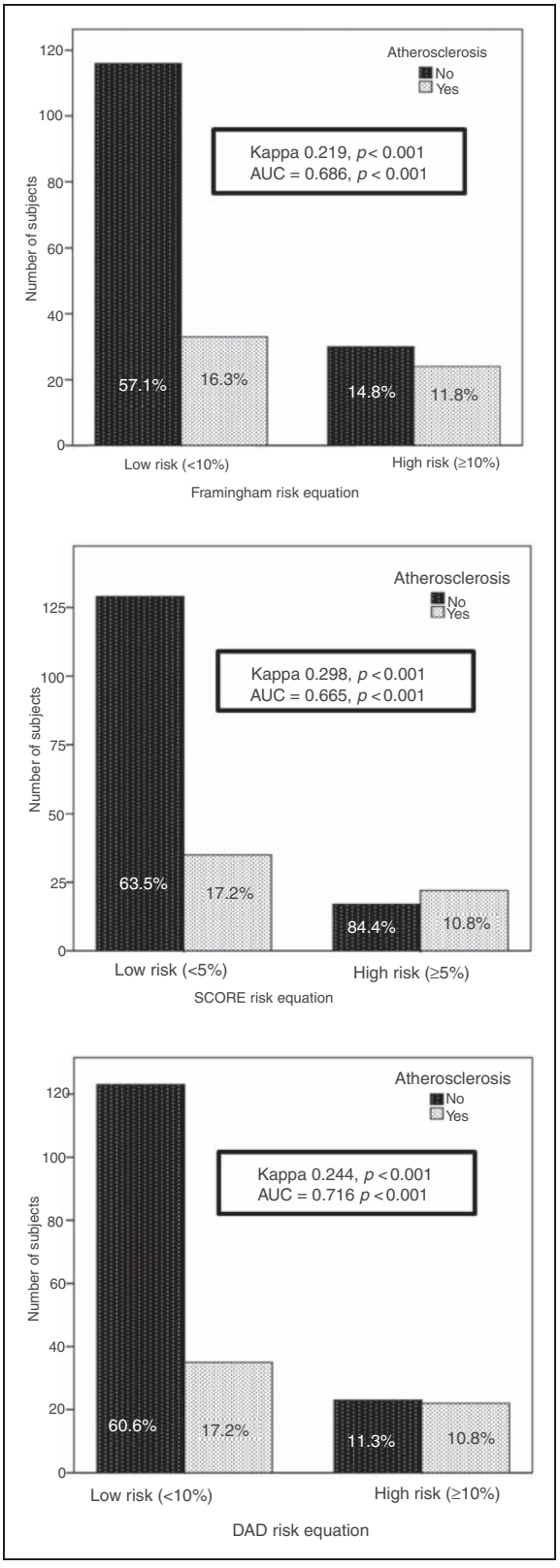

Figure 2. Agreement and global accuaracy between the estimation of cardiovascular disease risk based on the Framingham, SCORE and D:A:D equations and the observed subclinical atherosclerosis.

However, all three equations underestimate the CVD risk in 15–20% of patients.

CVD is currently among the leading causes of death in HIV-infected patients.[25] Therefore, as part of the global management of these subjects, the non-invasive detection of premature atherosclerosis must be prioritized. The American Heart Association guidelines advocate the use of the Framingham equation for the estimation of 10-year absolute risk of CVD in order to identify patients for more intensive preventive measures.[17] However, three main difficulties arise when approaching this issue in the context of HIV infection. First, the Framingham equation was developed in a US-based population[7] and overestimates the risk of CVD when applied to persons living in European countries.[26–28] Second, the Framingham equation underestimates the risk of coronary events in HIV-infected patients,[8] as well as the degree of subclinical atherosclerosis.[9] On the other hand, the recommended equation for European populations (SCORE)[10] has not been validated in HIV-infected subjects. Third, while HIV infection itself and its associated factors independently contribute to subclinical atherosclerosis,[29,30] both Framingham and SCORE equations only consider traditional cardiovascular risk factors and ignore HIV variables. Unlike Framingham and SCORE equations, which CVD risk prediction is based upon the relative weight of different cardiovascular risk factors, the recently proposed D:A:D was developed to overcome these difficulties, and considers the use of certain antiretroviral drugs, indinavir, lopinavir and abacavir, which have previously been associated with the risk of myocardial infarction.[31,32]

We examined the diagnostic performance of Framingham, SCORE and D:A:D equations for the diagnosis of subclinical atherosclerosis in our HIV-infected population, and the D:A:D equation was the most accurate, although all three equations showed only a moderate predictive performance (AUC = 0.686, 0.665, 0.716, respectively; $p = 0.048$). Consequently, the D:A:D equation better correlates with an emerging risk factor of CVD, the IMT, and this provides new evidence to recommend the D:A:D equation over Framingham and SCORE for the stratification of cardiovascular risk in HIV-infected subjects. Nonetheless, the presence of subclinical atherosclerosis remained underestimated, even with the D:A:D equation, in a significant proportion of patients (17.2%). This mismatch between the risk prediction and the observed presence of subclinical atherosclerosis could be explained by several reasons. Although the D:A:D study is a large prospective cohort designed to analyse the adverse effects of long-term exposure to antiretroviral therapy, the data analysis present very challenging

Table 2. D:A:D risk equation performance for the diagnosis of subclinical atherosclerosis when cardiovascular and HIV-related variables were added to the model by logistic regression

Prediction of subclinical atherosclerosis	AUC	ΔAUC	*p*-value
D:A:D	0.716 (0.643–0.790)		<0.001
+ NRTI	0.723 (0.647–0.798)	+1%	<0.001
+ NNRTI	0.694 (0.614–0.774)	0%	<0.001
+ PI	0.717 (0.644–0.791)	+0.1%	<0.001
+ CD4 cell count	0.737 (0.665–0.809)	+3%	<0.001
+ C Reactive Protein	0.702 (0.625–0.779)	−2%	<0.001
+ Albumin/creatinine urine ratio	0.760 (0.679–0.841)	+6.1%	<0.001
+ CD4, Albumin/creatinine urine ratio	0.772 (0.694–0.851)	+8%	<0.001

AUC, area under receiver operating characteristic curve; Δ AUC, percentage increase in area under receiver operating characteristic curve; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, non-nucleoside reverse transcriptase inhibitor; PI, protease inhibitors; NRTI, NNRTI, IP and C-reactive protein were introduced in the model as continuous variables. CD4 cell count was categorized by the cut-off of 250 cells/ml, albumin/creatinine urine ratio was categorized by the median (5 mg/g).

issues, as the presence of collinearity between the duration of HIV infection and the accumulated exposure to antiretroviral drugs, as well as the concurrence of many temporal and related confounding effects, which could lead to find apparent associations.[33] Remarkably, prospective studies designed to analyse the impact of antiretroviral therapy on the incidence of CVD have also an inherent limitation: as subjects develop HAART-induced lipid abnormalities or glucose intolerance, these conditions are treated by their physicians, with the subsequent confounding effect on the incidence of cardiovascular events. Thus, a channelling bias has been suggested as a potential explanation of unexpected associations between the use of certain drugs and increased risk of myocardial infarction. Moreover, while an increasing consistent body of evidence support the role of protease inhibitors in the development of CVD,[31,34,35] the role of abacavir, a variable integrated into the D:A:D equation, originated a great controversy. A recent meta-analysis does not support that abacavir-containing regimens carry a greater risk of major cardiovascular events compared with abacavir-sparing regimens.[36]

In addition, even in the general population, CVD risk equations present an inherent limitation: they do not consider alternative CVD risk factors, and 25% of cardiovascular events happen in subjects with low or intermediate predicted CVD risk.[37] Then, it has been suggested that the use of biomarkers along with the risk equations could more accurately stratify patients into high or low CVD risk.[38] Indeed, the American Heart Association suggests that carotid IMT could be used to identify persons at higher risk than that revealed by the major risk factors alone. This strategy might be particularly helpful in HIV-infected patients, as HIV infection entails a constellation of additional pro-atherogenic factors of exceptional complexity. Apart of the impact of HIV infection itself and the adverse effects of antiretroviral drugs,[29,30,39] other factors are receiving increasing attention, such as the high level of bacterial translocation which has been related to all-cause mortality[40] and the induced immune activation.[41] Therefore, we believe that HIV-infected patients would especially benefit from a CVD risk model including biomarkers of vascular damage, inflammation and possibly immune activation.

A previous report by Parra et al. found that the Framingham equation underestimated the presence of subclinical atherosclerosis in HIV-infected patients, especially in those with increased levels of markers of chronic oxidative stress and persistent inflammatory status: monocyte chemoattractant protein-1 and oxidized LDL.[9] Of note, in our study all three equations underestimated the risk of subclinical atherosclerosis in nearly one fifth of subjects, which is a relative high proportion of patients. In the group of patients with low predicted risk of CVD by D:A:D, we found associations with older age and a low CD4 counts. This observation suggests that in certain patients, older subjects and those with a poorer immune status, the D:A:D equation may be particularly biased. A strategy to overcome this limitation might be the use of other non-invasive tools for recognition of subclinical atherosclerosis, such as the IMT, which should be recommended in certain patients in clinical guidelines for decisions regarding the implementation of preventive therapy for cardiovascular events. Another advantage of IMT against these equations would be the ability to monitor the efficacy of preventive measures in patients at higher CVD risk, as a direct measure of the efficacy of therapy in inducing regression of atherosclerosis.[42] Nevertheless, our results suggest that the D:A:D

equation might be further calibrated by incorporating markers of immune status and vascular damage. The best D:A:D diagnostic performance was reached when CD4 counts and low-grade albuminuria were incorporated into the formula by logistic regression analysis. These findings are consistent with an association of immune suppression with more rapid progression of atherosclerosis.[43,44] On the other hand, the immune status may influence albuminuria,[45] and low-grade albuminuria has been associated with all-cause and cardiovascular mortality. Therefore, ACR measurement could improve the identification of patients living with HIV at increased risk of CVD. Altogether, the results of the present study suggest that the stratification of CVD risk in HIV-infected patients can be improved by measuring markers reflecting the immunologic status and the presence of subclinical vascular damage, and a prospective study in order to validate this strategy is warranted.

Our study has several limitations. First, although Framingham, SCORE and D:A:D equations were calibrated to predict the risk of cardiovascular events, we did not study the incidence of cardiovascular events over time, but the current IMT. Thus, our study was not designed to further calibrate these equations, but to study their agreement with a surrogate marker of cardiovascular events, IMT, and to improve their predictive values adjusting by immunovirological variables and cardiovascular biomarkers. Nevertheless, IMT has proved to be an important predictor of cardiovascular events in the general population[12,13] and is the test recommended by the American Heart Association for the assessment of the atherosclerotic burden and to identify persons at higher risk than that revealed by the major risk factors alone.[17] Second, we did not measure IMT in HIV-uninfected subjects, so we are unable to perform comparisons between infected and uninfected persons. However, the absence of controlled subjects does not invalidate our finding, and specially the assessment of concordance between Framingham, SCORE and D:A:D equations in HIV-infected patients and the attempts to adjust D:A:D equation to improve its accuracy for the diagnosis of subclinical atherosclerosis in the HIV population.

In conclusion, although the D:A:D equation predicts better than Framingham and SCORE equations, the presence of subclinical atherosclerosis, all three equations underestimate subclinical atherosclerosis in HIV-infected patients. However, including into the D:A:D equation a sensitive marker of vascular damage, low-grade albuminuria, and a variable reflecting the immune status, CD4 counts, significantly improves the global accuracy for predicting IMT and therefore CVD risk in this population.

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Conflict of interest

SS-V has received honoraria from Gilead. VE has received honoraria from Janssen Cilag for board membership, from Ferrer International for consultancy and grants from Abott, MSD and Gilead and from Boehringer Inghelheim and BMS for educational presentations. AF-C has received grants from MSD, Astra-Zeneca and Pfizer.

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Factores clínicos y biomarcadores asociados a lesión vascular subclínica en la infección por el virus de la inmunodeficiencia humana

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RESUMEN

Fundamento y objetivos: Los pacientes con VIH presentan aterosclerosis acelerada y la enfermedad cardiovascular es una de las principales causas de mortalidad de estos pacientes. Nuestro objetivo fue identificar biomarcadores y factores clínicos relacionados con la aterosclerosis carotídea en pacientes con VIH.

Pacientes y método: Estudio transversal en pacientes con VIH. Se determinó el grosor íntima-media carotídeo (GIMc) y diferentes biomarcadores séricos en 235 sujetos. Los pacientes con GIMc ≥ p75 y/o placa fueron diagnosticados de enfermedad vascular subclínica (EVS).

Resultados: Edad 46 (11) años. El GIMc medio fue 0,58 (0,13) mm. Sesenta y cinco (27,8%) pacientes presentaron EVS. En comparación con los pacientes sin EVS, estos mostraron mayor frecuencia de lipodistrofia (OR: 2,7; IC95%: 1,4-4,9) e inmunodepresión (OR: 2,5; IC95%: 1,1-5,8), mayor tiempo de diagnóstico del VIH (≥ p50 [10 años], OR: 1,4; IC95%: 1,1-2,9), exposición a análogos de nucleósidos (≥ p50 [132 meses], OR: 3,2; IC95%: 1,7-6) e inhibidores de proteasa (≥ p50 [24 meses], OR: 2,2; IC95%: 1,1-3,6). Mostraron mayores niveles de diferentes biomarcadores séricos, destacando el NT-proBNP (≥ p75 [72,6 pg/ml], OR: 2,0; IC95%: 1,0-4,1) y el cociente albúmina/creatinina en orina (≥ p50 [5 mg/g], OR: 3,8; IC95%: 1,3-11). Tras el análisis multivariado, la EVS se relacionó con la edad (≥ p50 [46 años], (OR: 6,6; IC95%: 2,2-19,5; p = 0,001), el tiempo de diagnóstico del VIH (≥ p50 [10 años], OR: 3,1; IC95%: 1,0-11,0; p = 0,044) y la inmunodepresión (OR: 2,8, IC95%: 1-8,3; p = 0,048).

Conclusiones: En pacientes con VIH, la edad, el tiempo de evolución de la infección y la inmunodepresión se relacionan independientemente con la EVS. Los pacientes con EVS presentaron niveles aumentados de biomarcadores de daño vascular, destacando el cociente albúmina/creatinina en orina y el NT-proBNP.

Clinical factors and biomarkers associated with subclinical atherosclerosis in the human immunodeficiency virus infection

Keywords:
Human immunodeficiency virus infection
Carotid atherosclerosis
Cardiovascular risk factors
Clinical markers

ABSTRACT

Background and objective: HIV-infected patients present accelerated cardiovascular disease (CVD) and CVD is among the most important causes of mortality in this population. We aimed to identify biomarkers and clinical factors associated with subclinical atherosclerosis in HIV-infected patients.

Patients and methods: Carotid intima-media thickness (cIMT) and cardiovascular biomarkers were measured in 235 HIV-infected patients. Individuals with a cIMT ≥ 75th percentile or plaque were classified as having subclinical atherosclerosis and compared with patients without subclinical atherosclerosis.

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Results: Age was 46 (11) years old. Mean cIMT was 0.58 (0.13) mm. Sixty-five (27.8%) patients had subclinical atherosclerosis. These subjects had more frequently lipodystrophy (OR: 2.7; CI95%: 1.4-4.9), immunosuppression (OR: 2.5; CI95%: 1.1-5.8), longer time to HIV diagnosis (≥ p50 [10 years], OR: 1.4; CI95%: 1.1-2.9), longer exposure to nucleoside analogues (≥ p50 [132 months], OR: 3.2; CI95%: 1.7-6) and to protease inhibitors (≥ p50 [24 months], OR: 2.2; CI95%: 1.1-3.6). They also showed higher levels of several biomarkers such as NT-proBNP (≥ p75 [72.6 pg/ml], OR: 2.0; CI95%: 1-4.1) and albumin/creatinine urine ratio (≥ p50 [5 mg/g], OR: 3.8; CI95%: 1.3-11). After the multivariate analysis, subclinical atherosclerosis was associated with age (OR: 6.6; CI95%: 2.2-19.5; *P* = .001), a longer time to HIV diagnosis (OR: 3.1; CI95%: 1.0-11.0; *P* = .044) and immunosuppression (OR: 2.8; CI95%: 1-8.3; *P* = .048).
Conclusions: Among HIV-infected patients, time to HIV diagnosis and immunosuppression were independently associated with subclinical atherosclerosis. Patients with subclinical atherosclerosis showed increased levels of vascular damage biomarkers, especially albumin/creatinine urine ratio and NT-proBNP.

Introducción

La supervivencia de los pacientes infectados por el VIH ha mejorado drásticamente desde la introducción en 1996 del tratamiento antirretroviral de gran actividad (TARGA). Si bien la mortalidad debida al síndrome de inmunodeficiencia adquirida (sida) ha disminuido, se ha observado un aumento relativo de la mortalidad asociada a enfermedades no definitorias de sida[1]. Se ha comprobado un aumento de casi el doble en la incidencia de infarto de miocardio respecto a población no infectada tras ajustar por factores de riesgo cardiovascular (FRCV)[2], y se ha demostrado la existencia de aterosclerosis acelerada en estos sujetos[3]. Por otro lado, debido a que la precisión de la ecuación de Framingham parece no ser satisfactoria cuando se aplica en pacientes con VIH[4], se está extendiendo el uso del grosor íntima-media carotídeo (GIMc) como marcador de riesgo cardiovascular (RCV), ya que ha demostrado predecir de manera fiable la incidencia de episodios cardiovasculares[5]. En un metaanálisis de 26 estudios, se demostró que los sujetos con VIH presentan mayor GIMc que la población general, aunque no se encontró una mayor prevalencia de placas carotídeas[6]. En España, las principales series con pacientes con VIH han demostrado asociación con factores relacionados con el VIH, como el TARGA[7], y se ha demostrado que el VIH comparte mecanismos patogénicos con la aterosclerosis[8,9].

Debido a la dificultad para estratificar el RCV en los sujetos con VIH[4,10], resulta también interesante conocer si existe asociación entre el GIMc y aquellos biomarcadores que reflejan la presencia de daño miocárdico (troponina I, creatincinasa-fracción miocárdica [CK-MB]), disfunción ventricular (*N-terminal pro brain natriuretic peptide* [NT-proBNP], «N-terminal propéptido natriurético cerebral»), trombosis (dímeros-D, fibrinógeno, homocisteína), insuficiencia renal (filtrado glomerular, cociente albúmina/creatinina en orina), inflamación (proteína C reactiva [PCR], interleucina-6) o inestabilidad de la placa de ateroma (lipoproteína ligada a fosfolipasa A2 [Lp-PLA2]), situaciones que sí han demostrado asociarse con incremento de RCV y mortalidad y que podrían tener utilidad para evaluar el RCV y predecir la existencia de aterosclerosis subclínica[10-14].

El objetivo de nuestro estudio fue esclarecer qué factores relacionados con la infección por VIH y qué biomarcadores de RCV se asocian con la presencia de aterosclerosis carotídea.

Métodos

Diseño del estudio, participantes y criterios de selección

Estudio observacional, transversal, en sujetos infectados por el VIH reclutados en las consultas de Enfermedades Infecciosas de un hospital terciario, entre 2009 y 2010. Los criterios de exclusión fueron: enfermedad cardiovascular conocida (ictus o infarto de miocardio), claudicación intermitente o insuficiencia renal crónica. El estudio fue aprobado por el Comité de Ética y todos los pacientes firmaron un consentimiento informado.

Variables clínicas y antropométricas

En toda la población del estudio se recogió la siguiente información: sexo, edad, antecedentes familiares de cardiopatía isquémica precoz (infarto de miocardio antes de los 55 años en varones y 65 años en mujeres), historia de tabaquismo y tratamiento para control de los FRCV (tratamiento actual con antihipertensivos, estatinas y otros hipolipidemiantes, antidiabéticos orales o insulina). La hipertensión arterial y el síndrome metabólico se definieron según los criterios del *Adult Treatment Panel III*[15]. La diabetes mellitus se diagnosticó según los criterios de la Sociedad Americana de Diabetes[16]. Se tomó la presión arterial tras reposo de al menos 15 minutos, con el paciente en decúbito supino, en 2 determinaciones separadas 10 minutos entre sí, anotando solo la segunda de ellas. Se diagnosticó la lipodistrofia mediante la escala de severidad de lipodistrofia de la cohorte HOPS[17].

Las medidas de laboratorio se realizaron en sangre recogida en ayunas de 12 horas y en una muestra de orina. Las concentraciones de glucosa, hemoglobina glucosilada, insulina, albúmina, creatinina, fibrinógeno, colesterol total y triglicéridos se determinaron por métodos enzimáticos estandarizados. El colesterol asociado a lipoproteínas de alta densidad (cHDL), apoA-I, apoB y lipoproteína(a) se determinaron mediante nefelometría (Sistema Vista®, Siemens Diagnostics, Deerfield, EE. UU.). Se determinó la presencia de insulinorresitencia mediante el cálculo del índice HOMA *(Homeostasis Model Assessment)* = insulina (μU/ml) × glucosa (mg/dl)/22,5. Las concentraciones de colesterol asociado a lipoproteínas de baja densidad (cLDL) se calcularon por el método Friedewald, salvo en pacientes con niveles de colesterol por encima de 400 mg/dl (cLDL = colesterol total - [triglicéridos/5] + cHDL).

El recuento de linfocitos CD4+ se realizó por citometría de flujo estándar y la carga viral de VIH-1 por reacción en cadena de la polimerasa (método Cobas TaqMan, Roche®, Branchburg, EE. UU.). La infección por VHC se diagnosticó mediante serología positiva para dicho virus por enzimoinmunoanálisis. La PCR ultrasensible y la cistatina C fueron determinadas por nefelometría (sistemas Vista® y BN-Prospect®, respectivamente, Siemens Diagnostics, Deerfield, EE. UU.), la interleucina-6 y la homocisteína por quimioluminiscencia (IMMULITE 2000®, Siemens Diagnostics, Deerfield, EE. UU.), los dímeros-D por turbidimetría (Beckman-Coulter, Münster, Alemania), la CK-MB y el NT-proBNP por inmunoanálisis LOCI (sistema Vista®, Siemens Diagnostics, Deerfield, EE. UU.) y la Lp-PLA2 por inmunoanálisis turbidimétrico (Lp-PLA2 control kit y diaDexus PLAC® Test Reagent Kit®, San Francisco, EE. UU.).

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Determinación del grosor íntima-media carotídeo

Dos técnicos previamente entrenados y con una experiencia similar determinaron el GIMc mediante ultrasonografía con transductor lineal de alta resolución de 12 MHz de frecuencia (modelo HD7, US-Philips®, Eindhoven, Países Bajos). Se siguieron las recomendaciones del consenso de Mannheim[18]. Las medidas se efectuaron en la pared más lejana de la carótida común, 1 cm proximalmente a la bifurcación. Se adquirieron imágenes de segmentos de al menos 10 mm en soporte informático y se utilizó un *software* de detección del GIMc previamente calibrado, programa QLab *(Advanced Quantification Software)*[19]. Mediante este programa se determinó la media del GIMc de más de 400 medidas realizadas sobre cada segmento estudiado. Para la detección de placa se efectuaron cortes longitudinales y transversales en el modo B a nivel de carótida común, bulbo y carótida interna y a continuación se utilizó el modo doppler-color. La placa se definió como una estructura focal invadiendo la luz arterial de al menos 0,5 mm o del 50% del valor del IMT circundante; también se consideró placa un grosor mayor de 1,5 mm medido desde la interfase media-adventicia a la interfase íntima-luz. Dado que el GIMc ha demostrado correlacionarse de manera lineal con la incidencia de eventos coronarios[5], se clasificó a los pacientes en 2 grupos: aquellos que presentaron un GIMc $\geq$ percentil 75 o en los que se demostró la presencia de placa se consideró que presentaban mayor RCV y se categorizaron como sujetos con enfermedad vascular subclínica (EVS). Los demás sujetos fueron incluidos en el grupo de pacientes sin EVS.

Análisis estadístico

Las variables cualitativas se presentan con su distribución de frecuencias. Las variables cuantitativas se resumen en su media y su desviación estándar (DE) y las variables que no siguen una distribución normal se expresan con mediana y rango intercuartílico (p25-p75).

Se estudió la relación lineal entre el GIMc y la edad como variables continuas mediante el coeficiente de correlación paramétrico de Pearson. Se evaluó la asociación entre variables cualitativas y la EVS mediante el test de Chi-cuadrado. Como medida de efecto se calculó la *odds ratio* (OR) junto a su intervalo de confianza al 95% (IC95%). Para las variables cuantitativas que se distribuyeron de manera normal se utilizó la prueba t de Student para grupos independientes. Se calcularon las diferencias de medias junto a sus IC95%. Para las variables que no se distribuyen de manera normal se utilizó el test no paramétrico U de Mann-Whitney. Se ajustó un modelo de regresión logística con el objetivo de identificar aquellas variables que se relacionaban de manera independiente con la EVS. Se introdujeron en el modelo aquellas variables que en el análisis univariado fueron estadísticamente significativas y/o aquellas clínicamente relevantes. Se realizó un análisis estratificado evaluando la modificación del efecto entre la edad y cada una de las variables seleccionadas para su introducción en el modelo, y la EVS. Se presentan las OR ajustadas junto a sus IC95%. Se introdujeron en el modelo las interacciones significativas identificadas en el análisis estratificado. Para todos los análisis, se rechazó la hipótesis nula cuando el nivel de significación estadística alcanzado fue $< 0{,}05$, para contrastes de 2 colas. Previamente al inicio del análisis, se realizó un estudio piloto para determinar el coeficiente de correlación intraclase interobservador, tanto para el GIMc como para la presencia o no de placa. Se estudiaron un total de 29 pacientes, en los que ambos técnicos demostraron un índice de correlación intraclase superior a 0,926 (IC95%: 0,849-0,965). El grado de concordancia para la presencia de placa calculado mediante el índice kappa fue de 0,782 (IC95%: 0,372-1,000). Ambos observadores identificaron solo en 3 casos la presencia de placa con un grado de acuerdo absoluto del 96,5% (28/29).

Resultados

La muestra de pacientes se compuso de 234 individuos con infección por VIH, con una edad media de 46,7 (11,2) años, mayoritariamente varones (85,9%). El 10,3% de los pacientes no habían iniciado TARGA. Respecto a los FRCV, existe una frecuencia destacable de tabaquismo (44,0%), con una hipertensión arterial del 16,7%, diabetes en el 9,8% y obesidad únicamente en el 2,7%. La frecuencia de hipercolesterolemia (colesterol LDL $>$ 130 mg/dl o colesterol noHDL $>$ 160 mg/dl) fue del 65,8%, y la de hipertrigliceridemia (triglicéridos $\geq$ 150 mg/dl) del 42,9%. El 12,4% cumplió criterios de síndrome metabólico. El 7,3% recibía estatinas y todos los pacientes hipertensos usaban inhibidores de la enzima conversiva de la angiotensina (IECA) o antagonistas del receptor de aldosterona II.

El GIMc se correlacionó positivamente con la edad ($r = 0{,}484$; $p < 0{,}001$). Según la ecuación de la recta de regresión, en los varones el GIMc aumentaba 0,0133 mm por año de edad ($r = 0{,}454$; $p < 0{,}001$), siendo en mujeres el incremento anual de GIMc de 0,0071 mm ($r = 0{,}757$; $p < 0{,}001$). La distribución de los valores de GIMc encontrados, distribuidos por edad y sexo, se resume en la tabla 1.

Se encontraron placas carotídeas en 13 casos (18%). La proporción de placas varió también según el grupo de edad (tabla 2), encontrándose en mayor medida en el grupo de 46-55 años (36,5% del total de placas, $p = 0{,}024$). La presencia de placas no se relacionó con el tabaquismo, la prevalencia de hipertensión arterial, diabetes mellitus ni lipodistrofia.

Sesenta y cinco sujetos (27,8%) presentaron EVS. Sus características generales se resumen en la tabla 3. Estos pacientes presentaron mayor edad, mayor perímetro de cintura y con mayor frecuencia hipertensión arterial, diabetes mellitus y síndrome metabólico, con significación estadística para todas estas diferencias. Todos los sujetos hipertensos se encontraban en tratamiento con IECA o antagonistas del receptor de aldosterona II. La

Tabla 1
Valores del grosor íntima-media carotídeo por rango de edad en 234 sujetos con infección por el virus de la inmunodeficiencia humana

Sexo	Grupo de edad (años)	N = 233	Media (mm)	Desviación estándar (mm)	Percentil 25 (mm)	Mediana (mm)	Percentil 75 (mm)
Varón (N = 201)	$\leq$ 35	25	0,48	0,05	0,44	0,49	0,52
	36-45	70	0,57	0,12	0,49	0,55	0,62
	46-55	72	0,58	0,10	0,51	0,57	0,64
	56-65	20	0,72	0,19	0,56	0,70	0,80
	$>$ 65	14	0,69	0,12	0,61	0,67	0,78
Mujer (N = 33)	$\leq$ 35	6	0,43	0,06	0,40	0,43	0,45
	36-45	11	0,54	0,07	0,51	0,53	0,58
	46-55	13	0,58	0,07	0,56	0,57	0,61
	56-65	2	0,60	0,02	0,58	0,60	0,61
	$>$ 65	1	0,72			0,72	

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Tabla 2
Frecuencia de presencia de placa por edad y sexo en 234 sujetos con infección por el virus de la inmunodeficiencia humana

Sexo	Grupo de edad (años)	N	Placa de ateroma, N (% de grupo de edad)	
			No	Sí
Varones (N = 200)	≤ 35	25	25 (100)	0
	36-45	70	68 (97,1)	2 (2,9)
	46-55	72	68 (94,4)	4 (5,6)
	56-65	20	18 (90,0)	2 (10,0)
	> 66	14	10 (71,4)	4 (28,6)
Mujeres (N = 33)	≤ 35	6	6 (100)	0
	36-45	11	11 (100)	0
	46-55	13	12 (92,3)	1 (7,7)
	56-65	2	2 (100)	0
	> 66	1	1 (100)	0

Tabla 3
Análisis comparativo de los pacientes con enfermedad vascular subclínica

	Sin EVS, %	Con EVS, %	p
N = 234, N (%)	169 (72,2)	65 (27,8)	
Edad (años)	44,3 (8,9)	53,9 (10,5)	< 0,001
Sexo			
Varón, N (%)	141 (83,4)	60 (92,3)	0,057
Mujer, N (%)	28 (16,6)	5 (7,7)	
En TARGA, N (%)	147 (87,0)	62 (96,9)	0,017
Tabaquismo, N (%)	76 (45,0)	27 (41,5)	0,373
Hipertensión arterial, N (%)	23 (13,6)	16 (24,6)	0,036
Uso de IECA/ARAII, N (%)	23 (13,6)	16 (24,6)	0,036
Diabetes mellitus, N (%)	10 (5,9)	13 (20,0)	0,006
Uso de antidiabéticos orales, N (%)	3 (1,8)	4 (6,3)	0,188
Uso de insulina, N (%)	3 (1,8)	3 (4,7)	0,236
Hipercolesterolemia, N (%)	109 (65,7)	45 (70,3)	0,502
Uso de estatinas, N (%)	9 (5,4)	8 (12,3)	0,151
Hipertrigliceridemia, N (%)	65 (38,9)	34 (53,1)	0,051
Síndrome metabólico, N (%)	15 (9,9)	12 (21,1)	0,032
AF cardiopatía isquémica precoz, N (%)	22 (14,1)	10 (6,0)	0,589
Coinfección por VHC, N (%)	42 (27,5)	17 (28,8)	0,843
Lipodistrofia, N (%)	42 (26,1)	30 (48,4)	0,001
Cintura (cm)	87,6 (10,4)	91,2 (8,5)	0,013
IMC (kg/m²)	23,7 (2,8)	24,2 (2,7)	0,220
Duración infección VIH (años)	10 (4-15)	13 (6-17,5)	0,021
Carga viral > 50 copias/ml, N (%)	48 (28,6)	12 (19,0)	0,095
Linfocitos CD4 (cél/μl)[a]	528 (383-725)	489 (325-699)	0,319
Nadir CD4 (cél/ml)[a]	278 (167-405)	245 (110-360)	0,045
CD4 < 250 cél/ml, N (%)	13 (7,8)	11 (17,2)	0,036
Duración TARGA (meses)[a]	58 (16-123)	102 (47,5-144)	0,002
Duración ITIAN (meses)[a]	104 (30-216)	212 (90-258)	< 0,001
Duración análogos timidínicos (meses)[a]	0 (0-50,25)	32 (0-80)	0,002
Duración ITINAN (meses)[a]	19 (0-47)	27 (0-81)	0,172
Duración IP (meses)[a]	11 (0-91,25)	38 (6-92)	0,029

Medidas expresadas como media (DE), salvo si se indica otra medida.
AF: antecedentes familiares; EVS: enfermedad vascular subclínica; IMC: índice de masa corporal; IP: inhibidores de la proteasa; ITIAN: inhibidores de la transcriptasa inversa análogos de nucleósidos; ITINAN: inhibidores de la transcriptasa inversa no análogos de nucleósidos; TARGA: tratamiento antirretroviral de gran actividad; VHC: virus de la hepatitis C.
[a] Expresado como mediana (rango intercuartílico).

proporción de pacientes con EVS fue mayor en varones que en mujeres, rozando la significación estadística. No se encontraron diferencias en la proporción de fumadores, el índice de masa corporal, el uso de estatinas o antidiabéticos.

Los pacientes con EVS se diferenciaron también en aquellas variables relacionadas con la infección por VIH. Se encontraban mayoritariamente en TARGA y presentaron mayor duración de la infección por VIH. El tiempo acumulado en TARGA fue también mayor, hallando diferencias significativas con un mayor tiempo acumulado de exposición a inhibidores de la transcriptasa inversa análogos de nucleósidos (ITIAN), a análogos timidínicos (AZT, d4T) y a inhibidores de la proteasa (IP). Finalmente, el grupo de estudio presentó una frecuencia significativamente superior de lipodistrofia e inmunosupresión, así como menor nadir de linfocitos CD4. No se encontraron diferencias entre ambos grupos en la carga viral ni la duración del tratamiento acumulado con inhibidores de la transcriptasa inversa no análogos de nucleósidos (ITINAN) ni en la prevalencia de coinfección por VHC.

Perfil glucémico, perfil lipídico y biomarcadores de daño vascular

La tabla 4 resume las diferencias encontradas en el perfil glucémico, lipídico y los biomarcadores de daño vascular. Los niveles de glucosa sérica, hemoglobina glucosilada, insulina e índice HOMA fueron similares en ambos grupos, excluyendo para este subanálisis a los pacientes diabéticos. Llamativamente, no encontramos diferencias en el perfil lipídico entre los 2 grupos de pacientes (colesterol, cHDL, cLDL, apolipoproteína A, apolipoproteína B, lipoproteína [a]); únicamente la frecuencia de hipertrigliceridemia fue superior en los sujetos con EVS, rozando la significación estadística (53,1 frente a 38,9%, p = 0,051). De los parámetros de función renal estudiados, estos pacientes presentaron mayor cociente albúmina/creatinina en orina (≥ p50 [5 mg/g] 57,1 frente a 29,2%; p < 0,001). Sin embargo, no se encontraron diferencias en los valores de creatinina ni en el filtrado glomerular estimado por la ecuación MDRD.

Se compararon también los niveles de distintos biomarcadores de inflamación, daño vascular y trombosis. Estos pacientes presentaron niveles superiores de PCR, CK-MB, dímeros-D y NT-proBNP. Sin embargo, no se encontraron diferencias estadísticamente significativas en los niveles de interleucina-6, Lp-PLA2, fibrinógeno ni homocisteína. No obstante, los niveles de Lp-PLA2 se encontraron aumentados por encima del valor de referencia (235 ng/ml) en el 39,3% del global de los individuos.

Para el análisis multivariado (tabla 5) se ajustó un modelo de regresión logística por aquellas variables estadística y/o clínicamente relevantes. En un paso previo, se realizó un análisis multivariado únicamente con las variables relacionadas con el VIH, observándose una pérdida del efecto de algunas de ellas. Finalmente, las variables introducidas en el modelo fueron: edad, sexo, hipertensión, diabetes, duración de la infección por VIH, tiempo de tratamiento acumulado con ITIAN y PCR. En el análisis bivariado se observó una modificación del efecto significativa (p = 0,030) entre la edad y los años de evolución del VIH, que se introdujo también en el análisis multivariado.

Tras el análisis multivariado, se mantuvo la interacción entre edad y tiempo de evolución del VIH, se mantuvo la demostrada en el análisis bivariado. En el grupo de pacientes más jóvenes (edad < 46 años), una mayor duración de la infección condicionó la presencia de EVS (OR: 3,4; IC95%: 1,1-11; p = 0,044). Sin embargo, este efecto del VIH desapareció en el grupo de individuos de mayor edad (≥ 46 años), en los que el aumento del GIMc pareció condicionado esencialmente por la mayor edad (OR: 0,7;

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Tabla 4
Variables analíticas relacionadas con la presencia de enfermedad vascular subclínica

	Sin EVS, %	Con EVS, %	p
N = 234, N (%)	170 (72,2)	65 (27,8)	
Glucosa (mg/dl)[a]	93,4 (11,1)	96,6 (13,9)	0,088
Hemoglobina glucosilada[a]	5,4 (0,4)	5,5 (0,6)	0,442
Insulina (μIU/ml)[a,b]	7,3 (3,4-13,2)	9,2 (4,5-17,3)	0,072
Resistencia a la insulina (HOMA)[a,b]	2,2 (1,1-3,3)	2,3 (1,5-4,8)	0,142
Creatinina (mg/dl)	1,1 (0,2)	1,2 (0,8)	0,109
FG (ml/min/1,73 m²)[a,b]	78,5 (1,1)	74,5 (13,7)	0,058
Cociente albúmina/creatinina en orina (mg/g)[a,b]	4 (3-7)	7 (4-16)	< 0,001
Perfil lipídico			
Colesterol (mg/dl)	186,7 (42,0)	189,2 (37,7)	0,654
cHDL (mg/dl)	50,3 (12,6)	49,2 (12,3)	0,721
cLDL (mg/dl)	105,7 (34,4)	107,6 (30,7)	0,566
Colesterol noHDL (mg/dl)	137,2 (41,1)	140,1 (35,2)	0,646
Triglicéridos (mg/dl)	128,0 (93-189)	155,0 (108-277,8)	0,191
Apolipoproteína (A) (mg/dl)	148,2 (33,8)	145,0 (30,6)	0,526
Apolipoproteína (B) (mg/dl)	93,9 (26,6)	95,7 (25,1)	0,663
Lipoproteína (a) (mg/dl)	12,8 (5,5-31,9)	10,9 (3,9-26,2)	0,506
Marcadores de inflamación/trombosis			
Proteína C reactiva (mg/l)	1,4 (0,6-3,5)	2,1 (0,8-4,8)	0,040
Interleucina-6 (pg/ml)	1,9 (1,9-2,6)	1,9 (1,9-3,3)	0,132
Lp-PLA2 (ng/ml)	235,5 (76,6)	233,7 (68,8)	0,569
Fibrinógeno (mg/dl)	320,1 (74,1)	323,1 (97,1)	0,823
Homocisteína (μmol/l)	10,7 (8,6-13,3)	11,5 (10,2-12,4)	0,114
Dímeros-D (ng/ml)	180 (84-320)	195 (75-466)	0,023
Troponina I	0,010 (0,002)	0,016 (0,044)	0,356
Creatincinasa-MB (ng/ml)	0,7 (0,1-1,5)	1,1 (0,6-1,91)	0,019
NT-proBNP (pg/ml)	36 (20-65)	43 (20-108)	0,037

Medidas expresadas como media (DE), salvo si se indica otra medida.
FG: filtrado glomerular estimado; Lp-PLA2: lipoproteína asociada a fosfolipasa ácida A2; NT-proBNP: N-terminal propéptido natriurético cerebral.
[a] Seleccionando a los sujetos no diabéticos.
[b] Expresado como mediana (rango intercuartílico).

Tabla 5
Modelo de regresión logística para las variables relacionadas con la presencia de enfermedades cardiovasculares

	Odds ratio	IC95%	p
Edad[a]	6,6	2,2-19,5	0,001
Sexo (varón)	2,1	0,7-6,2	0,202
Diabetes	2,2	0,8-6,2	0,130
Hipertensión arterial	0,9	0,4-2,2	0,876
Hipertrigliceridemia	1,6	0,8-3,2	0,185
Años de diagnóstico de VIH[b]	3,4	1,0-11,0	0,044
Interacción entre edad y años VIH			0,030
Linfocitos CD4 < 250 (cél/μl)	2,8	1,0-8,3	0,048
Tiempo en ITIAN	1,5	0,6-3,6	0,341
Tiempo en ITINAN	1,2	0,5-2,8	0,640
Proteína C reactiva > 1,6 mg/l	1,7	0,9-3,3	0,134

Variables categorizadas por la mediana: edad, 46 años; años de diagnóstico de VIH, 10 años.
Variables categorizadas por p75: tiempo en ITIAN, 247 meses; tiempo en IP, 24 meses.
IP: inhibidores de la proteasa; ITIAN: inhibidores de la transcriptasa inversa análogos de nucleósidos; ITINAN: inhibidores de la transcriptasa inversa no análogos de nucleósidos.
[a] Efecto de la edad en el grupo de pacientes con mayor antigüedad del diagnóstico de VIH.
[b] Efecto de la antigüedad del diagnóstico de VIH en el grupo de pacientes jóvenes.

IC95%: 0,2-1,8; p = 0,41). La presencia de inmunodepresión se asoció también a EVS (OR: 2,8; IC95%: 1,0-8,3).

Discusión

Hemos descrito los valores de GIMc en una amplia serie de pacientes con VIH y analizado los biomarcadores y factores asociados con la presencia de mayor GIMc, hallando que la edad, el tiempo de evolución del VIH y la inmunodepresión se relacionan independientemente con la EVS. Como biomarcadores asociados con la presencia de EVS, destacan el cociente albúmina/creatinina en orina y el NT-proBNP.

La fisiopatología de la infección por VIH es un fenómeno de gran complejidad, ya que al efecto proaterogénico de los FRCV tradicionales se suman los efectos directos del VIH sobre el endotelio vascular[20], la inmunoactivación crónica[21] o los efectos secundarios del TARGA[22], como la dislipidemia[23]. Estudios previos han demostrado que el VIH y el TARGA se asocian independientemente a mayor GIMc[6,24]. En España, diferentes trabajos han analizado el GIMc en sujetos con VIH. Un estudio transversal demostró que esta asociación entre GIMc y TARGA ocurre independientemente de la estratificación previa del riesgo cardiovascular según la ecuación de Framingham[7]. El TARGA, en concreto los IP, se ha asociado a otros marcadores subrogados de RCV como el índice tobillo-brazo[25]. Un estudio prospectivo encontró asociación independiente entre el GIMc y una mutación en un alelo de la proteína quimioatrayente de monocitos-1, una citoquina inflamatoria que promueve la migración de los monocitos al subendotelio y que además actúa como correceptor del VIH, demostrando que este comparte mecanismos patogénicos con la aterosclerosis[8]. En el análisis de seguimiento de este estudio, los autores hallaron asociación entre una mayor progresión del GIMc y polimorfismos en algunos genes implicados en estas vías de la aterosclerosis, así como con un recuento bajo de linfocitos CD4[9]. Aunque se ha prestado más atención al efecto del TARGA sobre el RCV que al papel de la inmunodepresión, es posible que cuando esta es crónica sea más nociva a nivel cardiovascular que el propio TARGA. En un estudio multicéntrico de casos y controles con casi 2.000 pacientes con VIH, se encontró que el recuento bajo de linfocitos CD4 fue la variable asociada con mayor consistencia a la aterosclerosis carotídea[26]. Si bien las relaciones fisiopatológicas entre inmunosupresión y aterosclerosis están por aclarar, es probable que desempeñen un papel central la activación

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inmune y la traslocación bacteriana crónicas asociadas a la infección por VIH[27]. Son necesarios más estudios para aclarar estas relaciones.

Resulta también interesante estudiar los biomarcadores asociados a la EVS en una población de mayor RCV como son los pacientes con VIH, debido a que más de la mitad de los episodios coronarios ocurren en sujetos sin FRCV[28]. En nuestro estudio analizamos un extenso panel de biomarcadores relacionados con la inflamación y la trombosis crónicas, que en diferentes trabajos se habían asociado a enfermedad cardiovascular, algunos de ellos poco estudiados en el contexto de la infección por VIH.

La PCR es el marcador de RCV con un mayor cuerpo de evidencia para su uso como predictor de eventos cardiovasculares. Se ha observado que los sujetos con VIH presentan mayor GIMc que aquellos no infectados, incluso en ausencia de replicación viral o de TARGA, y que esto se relaciona con niveles aumentados de PCR[29]. En este estudio, los pacientes con EVS presentaron niveles superiores de PCR, aunque estas diferencias desaparecieron tras el análisis multivariado. Los valores de PCR hallados en nuestra muestra sí se han asociado en la población general a un incremento moderado del RCV[30]. Sería interesante estudiar prospectivamente si la PCR se relaciona con una mayor progresión del GIMc en sujetos con VIH.

Respecto a la Lp-PLA2, una enzima específica de inflamación vascular implicada en la formación de placas de ateroma inestables que es objeto de intensa investigación en la actualidad[31], no hemos encontrado asociación con la presencia de aterosclerosis carotídea. Pocos trabajos han buscado asociación entre los niveles de Lp-PLA2 y la aterosclerosis carotídea y, hasta donde conocemos, ninguno en sujetos infectados por el VIH. En nuestro estudio, no hallamos diferencias entre ambos grupos de pacientes. Creemos que esta ausencia de relación puede deberse a que la Lp-PLA2 es más un marcador de la calidad de la placa de ateroma que de la carga aterosclerótica total[32] y los sujetos de nuestro estudio, de edad media y sin enfermedad cardiovascular conocida, posiblemente se hallen en una fase relativamente precoz del proceso aterosclerótico.

Otros marcadores que se han relacionado en nuestro estudio con el GIMc han sido los dímeros-D (marcador de trombosis), la CK-MB (marcador de necrosis miocárdica), el cociente albúmina/creatinina en orina (marcador de alteración en la microvasculatura glomerular renal) y el NT-proBNP (marcador de sobrecarga ventricular izquierda). Estos biomarcadores, o bien reflejan diferentes consecuencias del proceso aterosclerótico, o bien pueden estar relacionados con factores predisponentes de base. Es importante el estudio de biomarcadores para identificar a los sujetos con mayor RCV, y en este sentido creemos que, a la luz de nuestros datos, el cociente albúmina/creatinina en orina y el NT-proBNP son los que podrían resultar más rentables.

Conviene remarcar la interacción observada entre la edad y la antigüedad de la infección por VIH, que permaneció significativa tras el análisis multivariado, ya que no hemos encontrado alusión a la misma en estudios de diseño similar. El efecto aterogénico de la infección por VIH se tradujo en una mayor frecuencia de aterosclerosis carotídea en los pacientes más jóvenes; sin embargo, dicho efecto no fue significativo en el grupo de mayor edad. Esto sugiere que es posible que el VIH ocasione el mayor efecto sobre el proceso aterosclerótico en los primeros años de la infección, en el contexto prooxidativo e inflamatorio que caracteriza a la infección por VIH sin TARGA.

Este estudio tiene varias implicaciones clínicas. En primer lugar, describe los valores de GIMc hallados en una amplia serie de pacientes con VIH sin enfermedad cardiovascular en España, lo que puede ser de utilidad para estudios comparativos. En segundo lugar, identifica las variables relacionas con la aterosclerosis carotídea de forma independiente en estos sujetos, lo que sugiere que los pacientes con menor número de linfocitos CD4 y aquellos con larga duración de la infección por VIH deberían ser objeto del mayor esfuerzo en vigilancia del RCV. En tercer lugar, describimos asociaciones entre la aterosclerosis carotídea y diferentes biomarcadores séricos, que pueden ayudar a diseñar herramientas de estratificación del RCV en los pacientes con VIH en un futuro próximo; sugerimos que el uso del cociente albúmina/creatinina y el NT-proBNP puede ser de especial utilidad.

Nuestro estudio está sujeto a una serie de limitaciones. La principal es el diseño transversal, que imposibilita demostrar relaciones de causalidad. Otra limitación es el no utilizar valores de referencia poblacionales para la definición de aterosclerosis carotídea. Sin embargo, se tuvieron en cuenta las siguientes consideraciones antes de decidir EVS a través del p75 del GIMc o la presencia de placa: en primer lugar, el GIMc se correlaciona con la incidencia de episodios cardiovasculares sin efecto umbral[5]. En segundo lugar, el GIMc depende en gran medida de la edad, y la dispersión en la variable edad de nuestra muestra fue pequeña. En tercer lugar, el p75 indica RCV aumentado según el Grupo de Grosor Íntima-Media Carotídeo de la Sociedad Americana de Ecocardiografía[33]. Creemos que el hecho de usar percentiles asegura que identificamos 2 grupos homogéneos de pacientes de mayor y menor RCV.

El hecho de no haber encontrado diferencias entre ambos grupos en el perfil lipídico, salvo en los triglicéridos, sugiere que es probable que se haya producido un sesgo de canalización debido al diseño transversal del estudio. La dislipidemia ocasionada por los fármacos antirretrovirales de primera generación al inicio de la epidemia por VIH pudo favorecer durante años la progresión de la aterosclerosis carotídea. A estos pacientes, con mayores alteraciones lipídicas, probablemente se les modificó el tratamiento por esquemas de TARGA con menor toxicidad metabólica, no pudiendo detectarse este hecho con un estudio transversal. Finalmente, el limitado tamaño muestral y la frecuencia de aparición de la variable dependiente imposibilitó incluir en el análisis multivariado variables que habría sido interesante analizar, como algunos biomarcadores séricos.

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Conflicto de intereses

Los autores declaran no tener ningún conflicto de intereses.

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Incipient Renal Impairment as a Predictor of Subclinical Atherosclerosis in HIV-Infected Patients

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Introduction: HIV-infected patients present increased incidence of cardiovascular disease (CVD). Although incipient kidney function impairment has been associated with CVD in the general population, this association has not been properly addressed in HIV-infected patients. We assessed the relationship between incipient renal impairment (IRI) and subclinical atherosclerosis in HIV-infected patients.

Methods: Estimated glomerular filtration rate (eGFR), carotid intima-media thickness (cIMT), and cardiovascular biomarkers were measured in 145 HIV-infected patients. IRI was defined as a composite variable: eGFR <90 mL/min, rate of eGFR decrease >3% annually over a period of 3 years, and albumin/creatinine urine ratio above the median (≥5 mg/g). Individuals with a cIMT ≥75th percentile or plaque were classified as having subclinical atherosclerosis.

Results: Ninety-five patients (64.1%) met the criteria for IRI. As for HIV-related factors, patients with IRI more frequently had lipodystrophy (41.3% vs. 21.6%; $P = 0.017$), a lower CD4 lymphocyte nadir [210 (125–343) vs. 302 (178–408) cells/mL; $P = 0.046$], and longer exposure to nucleoside reverse transcriptase inhibitors [187 (84–259) vs. 104 (34–170) months; $P = 0.001$], to nonnucleoside reverse transcriptase inhibitors [32 (7–77) vs. 20 0–40) months; $P = 0.043$], and to protease inhibitors [42 (0–115] vs. 2.5 (0-59) months; $P = 0.007$]. Patients with IRI more frequently had subclinical atherosclerosis (40.7% vs. 13.7%; odds ratio: 4.3; 95% confidence interval: 1.8 to 10.6; $P = 0.001$), even after adjustment for cardiovascular and HIV-related parameters (odds ratio: 3.8; 95% confidence interval: 1.3 to 11; $P = 0.012$).

Conclusions: The presence of IRI is an independent predictor of increased cIMT in HIV-infected patients and may help to identify patients with subclinical atherosclerosis and, therefore, increased risk of CVD.

Key Words: carotid atherosclerosis, cardiovascular risk factors, clinical markers, HIV infection, renal impairment

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INTRODUCTION

More than a decade after the introduction of highly active antiretroviral therapy (HAART) in 1996, concern has been raised regarding the renal effects of long-term HIV infection and its treatment. Although HAART has improved survival considerably, patients are at increased risk of chronic kidney disease due to a number of factors, including ageing and HAART-related metabolic complications—hypertension,[1] diabetes mellitus,[2] dyslipidemia[3]—and the adverse effects of some antiretroviral drugs, especially tenofovir.[4,5] Indeed, kidney function is abnormal in up to 30% of HIV-infected patients, and kidney disease may be associated with progression to AIDS and death.[6–8] In addition, an independent association between decreased kidney function and the risk of cardiovascular events has been demonstrated in HIV-infected patients.[9] Most studies have investigated the association between the presence of cardiovascular disease (CVD) and impaired kidney function using

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the values for estimated glomerular filtration rate (eGFR) established for chronic kidney disease in the US National Kidney Foundation Guidelines (<60 mL·min^{-1}·1.73 m^{-2})[10]; however, most patients experience only mild renal function abnormalities whose clinical significance has yet to be defined.[11] Importantly, in the general population, the presence of low-grade albuminuria, a low eGFR, and a rapid decline in kidney function have been associated with all-cause and cardiovascular mortality.[6,12–14]

The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) recently developed a formula to calculate the eGFR–EPI equation, which has proved to be more accurate than the routinely used modification of diet in renal disease (MDRD) formula, especially in patients with normal kidney function.[15–17]

In this context, the early and accelerated atherosclerotic process described in HIV-infected patients[18] may be an additional contributing factor in kidney disease. Prompt diagnosis of subclinical atherosclerosis may enable aggressive management of contributing factors before the onset of clinical CVD. Several noninvasive approaches have been suggested to detect patients at increased risk of CVD, including measurement of carotid intima-media thickness (cIMT), which has proved to be an important predictor of cardiovascular events in both the general population[19,20] and in HIV-infected patients.[21]

Given that mild renal abnormalities have been associated with an increased incidence of cardiovascular events in community-based populations[12–14] and that indirect measurement of subclinical atherosclerosis such as cIMT has demonstrated a relationship between these abnormalities and cardiovascular events,[19,20] we assessed the hypothesis that the presence of incipient alterations in renal function could independently predict the presence of subclinical atherosclerosis in HIV-infected patients.

METHODS

Study Design, Participants, Setting, and Eligibility

We conducted an observational cross-sectional study of 145 consecutive HIV-infected patients who attended our university based HIV clinics in a tertiary hospital in Madrid between 2009 and 2010. The exclusion criteria were known CVD (previous stroke, myocardial infarction, or intermittent claudication) and/or known chronic kidney disease. The local ethics committee approved the study, and all patients gave their written informed consent to participate.

Clinical and Laboratory Measurements

Medical records were carefully reviewed at interview, a questionnaire was completed, and a thorough physical examination was performed. Gender, age, body mass index, smoking status, family history of early CVD, and treatment with antiretroviral drugs were recorded. The presence of hypertension, hypercholesterolemia, hypertriglyceridemia, and metabolic syndrome was defined according to the Adult Treatment Panel III criteria.[22] Lipodystrophy was defined as the presence of body fat changes that could be clearly recognized by the patient and confirmed by the doctor and diagnosed following the lipodystrophy severity grading scale of Lichtenstein et al.[23]

A sample of fasting venous blood was obtained to determine concentrations of glucose, insulin, interleukin-6, total cholesterol, high-density lipoprotein cholesterol, and triglycerides using standard enzymatic methods. Insulin resistance was calculated with the following equation: HOMA (homeostasis model assessment) = insulin (μU/mL) x glucose (mg/dL)/22.5. Low-density lipoprotein cholesterol concentrations were calculated using the Friedewald equation.[24]

Plasma viral load was measured using the Cobas TaqMan HIV-1 assay (Roche Diagnostics Systems, Inc, Branchburg, NJ), and CD4 lymphocyte count was determined by flow cytometry (Beckman-Coulter, Inc, Münster, Germany). Hepatitis C virus coinfection was diagnosed by a positive serology result using a standard enzyme-linked immunosorbent assay.

Plasma levels of high-sensitivity C-reactive protein (CRP) were measured using nephelometry (VISTA System, Siemens Healthcare Diagnostics Inc, Deerfield, IL). D-dimers were measured using turbidimetry (Beckman-Coulter, Inc, Münster, Germany) and fibrinogen concentration using a prothrombin time–derived method. N-terminal pro-B–type natriuretic peptide was measured using a luminescent oxygen-channeling assay (VISTA System, Siemens Healthcare Diagnostics Inc).

Renal Function Assessment

One first morning urine sample was collected from each participant and urinary albumin and creatinine concentrations were determined by turbidimetry (Olympus Diagnostics AU2700 autoanalyzer). Creatinine was measured using the kinetic Jaffe method (Olympus Diagnostics AU2700 autoanalyzer), and C-cystatin was analyzed by nephelometry (BN-Prospect System, Siemens Diagnostics). Albuminuria was calculated using the albumin/creatinine urine ratio (ACR) measured in 1 urine sample.

eGFR was calculated using the CKD-EPI formula.[16] The decrease in eGFR was determined by calculating the difference in eGFR value 3 years before the onset of the study and at the onset of the study. Given the absence of a standardized definition of IRI, several definitions were analyzed using data from studies that had previously explored the association between IRI and CVD.[12–14] The IRI definitions assessed were as follows: "A" = stage 2 chronic kidney disease (eGFR between 60 and 90 mL/min)[10] ± rapid decline in eGFR (>3% per year); "B" = "A" ± ACR >5 mg/g (median); "C" = "A" ± ACR >10 mg/g; "D" = "A" ± ACR >30 mg/g.[12,13,21,22]

Measurement of cIMT

cIMT measurements were obtained by ultrasonography (HD7 model, US Philips) according to the Mannheim Criteria.[25] Measurements were made at the common carotid artery (1 cm proximal to the bifurcation). Far wall cIMT images were obtained and digitalized for each patient. cIMT detection software was previously calibrated using QLab (Advanced Quantification Software).[26] More than

400 measurements were performed for each patient, and the median value was used for the statistical analyses. A plaque was defined as a thickness >1.5 mm or a focal structure that encroached into the arterial lumen by at least 0.5 mm or at least 50% of the surrounding cIMT value. Participants were categorized in 2 groups: those who presented a cIMT ≥ 75th percentile or in whom a plaque was demonstrated were considered to be at increased CVD risk and were classified as having subclinical atherosclerosis; the remaining patients were included in the comparison group. Several factors were taken into consideration before cIMT was classified as being above the 75th percentile. First, cIMT correlates with the incidence of cardiovascular events, and no threshold effect is observed.[20] Second, cIMT depends largely on age; however, reference values of cIMT adjusted for age are lacking in our population. Third, in our study, the dispersion in mean age was slight. Finally, the 75th percentile is indicative of increased CVD risk according to the American Society of Echocardiography cIMT Task Force.[27] Measurements were performed by 2 trained technicians who had previously participated in a pilot study (repeated and blinded measurements performed in 29 patients). The intraclass correlation coefficient was >0.90.

Statistical Analysis

Qualitative variables were summarized as a frequency distribution and normally distributed quantitative variables as mean ± standard deviation. The continuous nonnormally distributed variables were summarized as median and interquartile range. Means for variables with a normal distribution were compared using the *t* test. Nonparametric variables were studied using the Mann–Whitney test. Regression analysis and the Pearson correlation coefficient were used to evaluate the relationship between cIMT and eGFR. The Spearman rank correlation was used to evaluate the relationship between cIMT and ACR in first morning urine samples. A logarithmic transformation of the raw ACR data was applied to meet the requirements of regression analysis. Patients with and without IRI were compared using the χ^2 test or Fisher exact test when more than 25% of the expected values were less than 5. Logistic regression analysis was used to study the variables associated with the presence of subclinical atherosclerosis (as a binary variable).

Of the 4 IRI definitions proposed, we selected the one which best classified patients according to their degree of atherosclerosis by calculating the sensitivity, specificity, and area under the curve using a logistic regression model.

A logistic regression model was built to evaluate the effect of IRI on the degree of subclinical atherosclerosis. The variables included in the model were those that had a *P* value <0.10 and/or were clinically relevant between patients with and without IRI. The adjustment strategy involved calculation of the crude odds ratio of the association between the presence of IRI and the degree of subclinical atherosclerosis followed by individual adjustment of the effect of each potential confounding variable using a series of bivariate models. If any of these variables led to a change in the initial odds ratio for the presence of IRI >10%, they were included in a multivariate model (full model). Finally, the adjusted odds ratio (OR) and its 95% confidence interval (95% CI) were calculated. The null hypothesis was rejected by a type I error <0.05 ($\alpha < 0.05$). Statistical analyses were performed using SPSS 15.0.

RESULTS

Table 1 summarizes the characteristics of the 145 HIV-infected participants included in the study. The study sample was representative of a middle-aged (41 ± 10 years) HIV-positive population, and the most frequent cardiovascular risk factor was smoking (46.5%), followed by hypertriglyceridemia (43.4%), hypercholesterolemia (38.6%), hypertension (16.6%), and diabetes mellitus (9.0%). Metabolic syndrome was detected in 12.1% of patients. Most patients were on HAART (91.4%) and had an undetectable viral load (77.1%).

Mean cIMT was 0.59 (0.13) mm [median: 0.56 (0.50–0.65) mm]. The association between cIMT as a continuous variable with eGFR and ACR is shown in Figures 1, 2. cIMT correlated inversely with eGFR ($r = -0.381$, $P < 0.001$, Pearson correlation) and positively with log ACR ($r = 0.35$, $P < 0.001$, Spearman correlation). The presence of plaque was demonstrated in 11 (6.4%) cases, and 44 individuals (31.0%) were classed as having subclinical atherosclerosis. Although patients with subclinical atherosclerosis had a lower eGFR and a higher ACR than patients without subclinical atherosclerosis, both groups showed normal eGFR and ACR below the threshold of microalbuminuria (30 mg/g) (Table 2). No differences were found for creatinine and C-cystatin.

Table 3 summarizes the sensitivity, specificity, and area under the curve of the 4 different definitions of IRI for the diagnosis of subclinical atherosclerosis. The definition that best classified patients was "B".

Association Between IRI and Subclinical Atherosclerosis

A total of 95 patients (64.1%) presented IRI according to definition "B". Table 1 summarizes the characteristics of these patients.

HIV patients with IRI were older and had a higher waist circumference than patients without IRI. Hypertriglyceridemia, diabetes, and metabolic syndrome were more frequent in patients with IRI. Regarding HIV-related variables, patients with IRI more frequently had lipodystrophy, a lower CD4 lymphocyte count, lower CD4 lymphocyte nadir, undetectable viral load, and longer exposure to antiretroviral therapy. However, IRI was not associated with accumulated exposure to tenofovir or with ongoing treatment with tenofovir.

In the univariate analysis, the presence of IRI was statistically associated with the presence of subclinical atherosclerosis (OR: 4.3; 95% CI: 1.7 to 10.6; $P = 0.001$). Subsequently, a logistic regression model was built to analyze the presence of IRI as an independent predictor of subclinical atherosclerosis. We examined the impact of a number of potential confounding variables (age, diabetes mellitus, hypertension, hypertriglyceridemia, time to HIV

TABLE 1. Characteristics of the Study Population and Differences in Patients With and Without Incipient Renal Impairment

	All	No Incipient Renal Impairment	Incipient Renal Impairment	*P*
N, %	145, 100	52, 35.9	93, 64.1	—
Subclinical atherosclerosis, %	31	13.7	40.7	0.001
Age (yrs)	41.2 ± 10.3	40.7 ± 7.1	50.8 ± 10.0	<0.001
Male, %	87.6	84.6	89.2	0.417
Waist (cm)	88.3 ± 9.9	85.9 ± 9.8	89.6 ± 9.7	0.038
BMI (kg/m^2)	23.6 ± 2.7	23.3 ± 2.6	23.7 ± 2.8	0.401
Smokers, %	46.5	53.8	42.4	0.125
Hypertension, %	16.6	11.5	19.4	0.225
Diabetes mellitus, %	9.0	1.9	12.9	0.032
Hypercholesterolemia, %	38.6	37.3	43	0.486
Hypertriglyceridemia, %	43.4	31.4	52.1	0.013
Family history of early CVD, %	10.6	13.8	10.5	0.313
Metabolic syndrome, %	12.1	4	16.7	0.031
Use of ACEI or ARB, %	16.6	11.5	19.4	0.225
Lipid profile				
Cholesterol (mg/dL)	185.8 ± 41.1	187.6 ± 42.9	184.7 ± 40.3	0.687
HDL cholesterol (mg/dL)	50.1 ± 12.6	50 ± 12.2	50.1 ± 12.8	0.968
LDL cholesterol (mg/dL)	106.4 ± 37.3	106.5 ± 35.4	105.8 ± 38.5	0.805
Triglycerides (mg/dL)	161.6 ± 100.4	153.6 ± 108.8	166.1 ± 95.8	0.064
Lipodystrophy, %	34.3	21.6	41.3	0.017
CD4 nadir (cell/mL)*	265 (136–374)	302 (178–415.5)	210 (125–344)	0.046
Nadir CD4/CD8 ratio*	14.0 (8.0–21)	18.7 (10.4–25.4)	12.2 (6.7–18.2)	0.005
Undetectable viral load, %	77.1	67.3	82.6	0.036
On antiretroviral therapy, %	88.9	84.6	96.8	0.017
Time to HIV diagnosis (yrs)*	11 (5–16)	8 (4–13.75)	12 (6–18)	0.010
Exposure to NRTI (mo)*	145 (58.5–251.3)	104 (34–170)	187 (84–259)	0.001
Exposure to thymidine analogues (mo)*	10 (0–63)	0 (0–38)	29 (0–83)	0.003
Exposure to tenofovir (mo)*	37.5 (14–52)	33 (15–48)	39 (14–53)	0.493
Ongoing treatment with tenofovir, %	64.6	63.5	65.2	0.832
Exposure to NNRTI (mo)*	24 (0–63)	20 (0–40)	32 (7–77)	0.043
Exposure to PI (mo)*	32 (0–93.5)	2.5 (0–59)	42 (0–115)	0.007
Hepatitis C coinfection, %	25.8	34.1	22.5	0.231

IRI defined as a composite variable: eGFR between 60 and 90 mL/min, rate of eGFR decrease >3% annually over a period of 3 years, and ACR above the median (≥5 mg/g).
All values are expressed as mean ± SD unless otherwise specified.
*Expressed as median (IQR).
BMI, body mass index; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NRTI, nucleoside reverse transcriptase inhibitors; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitors.

diagnosis, accumulated exposure to antiretroviral drugs, CD4 lymphocyte count, detectable viral load, lipodystrophy, and CRP levels) by constructing a series of bivariate models according to the strategy previously described. Finally, we built the "full model" including the following confounding variables: age, diabetes mellitus, use of angiotensin-converting enzyme inhibitors or angiotensin receptor blockers, and accumulated exposure to nonnucleoside reverse transcriptase inhibitors and protease inhibitors. Multivariate analysis showed the presence of IRI to be independently associated with subclinical atherosclerosis (OR: 3.8; 95% CI: 1.3 to 11.0; $P = 0.013$).

Cardiovascular Biomarkers and IRI

HIV-infected patients with IRI tended to show higher levels of circulating cardiovascular biomarkers (CRP, fibrinogen, D-dimers, N-terminal pro-B–type natriuretic peptide) than patients without IRI; however, these increments were not statistically significant (Table 4).

DISCUSSIONS

Principal Findings and Comparisons With the Literature

Although several studies have demonstrated that HIV-infected patients have thicker cIMT than noninfected individuals, the factors associated with subclinical atherosclerosis in these patients remain unclear. In addition, the presence of IRI is common in these individuals and has proved to be an independent marker of cardiovascular events in the general population[7,12,13]; however, to our knowledge, its association with subclinical atherosclerosis has not yet been investigated

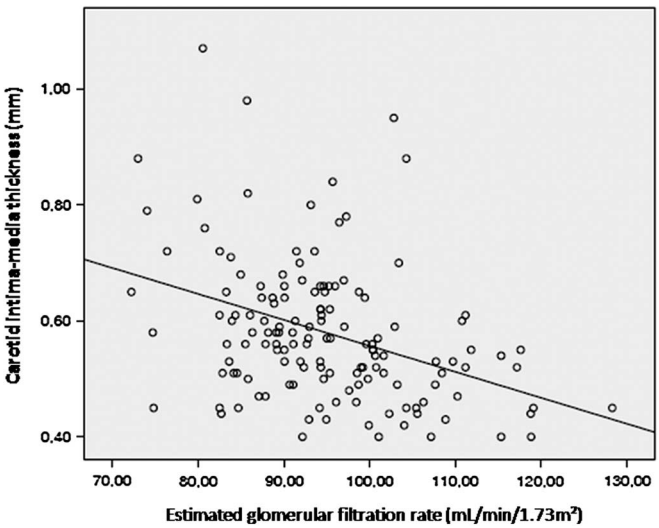

FIGURE 1. Regression linear analysis for the association between cIMT and the eGFR ($r = -0.381$, $P < 0.001$, Pearson correlation).

in HIV-infected patients. In our study, individuals with IRI, as determined by slight decreases in eGFR, a rapid decline in kidney function and/or low-grade albuminuria, had a 4-fold higher risk of subclinical atherosclerosis. Importantly, this increased risk was present at levels below the current threshold for microalbuminuria.

Current Infectious Diseases Society of America guidelines advocate evaluation of both eGFR and proteinuria when assessing renal function in HIV-infected patients.[28] The Infectious Diseases Society of America recommends using eGFR measured by serum creatinine–based equations in routine clinical practice, mainly the MDRD equation. However, serum creatinine among HIV-infected persons can vary considerably according to body mass, associated metabolic abnormalities, and exposure to drugs that affect renal tubular

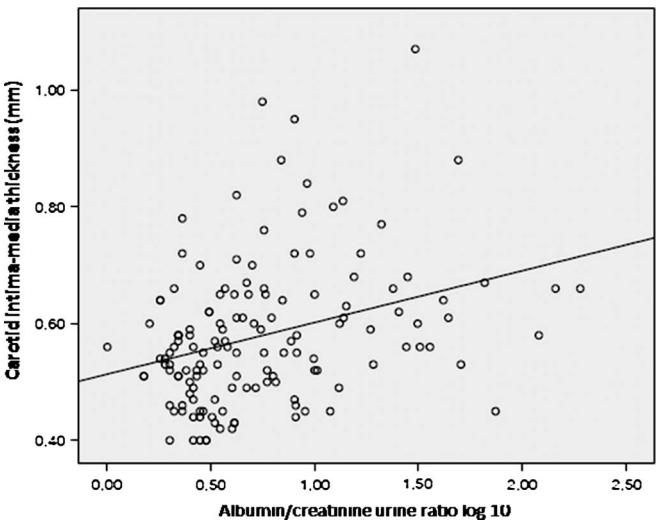

FIGURE 2. Regression linear analysis for the association between cIMT and the ACR after logarithmic transformation ($r = 0.35$, $P < 0.001$, Spearman correlation).

creatinine secretion.[29,30] Furthermore, the MDRD equation tend to underestimate actual GFR in subjects with normal or only mildly impaired kidney function,[31,32] as is the case in most HIV-infected patients.[11] More recently, the CKD-EPI equation has proved to provide GFR estimates with better accuracy and less bias than the MDRD equation[16,33] and, in contrast to the MDRD equation, eGFR >60 ml/min/1.73 m^2 can be calculated accurately using the CKD-EPI equation.[34] Although a growing body of evidence supports the use of this new equation, results should be confirmed in larger groups; however, for our study, it seemed to be the most appropriate estimator of eGFR, as we aimed to detect patients in the earliest stages of kidney disease.

Previous reports on patients with proteinuria clearly show that the presence of microalbuminuria (ACR >30 mg/g or a positive urine dipstick result) is associated with an increased risk of death.[35,36] In our study, the median ACR value was low (5 mg/g). As mentioned above, the presence of low-grade albuminuria, a low eGFR, and a rapid decline in kidney function have also been associated with all-cause and cardiovascular mortality in community-based populations.[6,12–14] In this study, we demonstrated a relationship between the presence of IRI and increased cIMT, a validated surrogate marker of cardiovascular events in HIV-infected patients.

Possible Mechanisms for the Observed Association

We suggest that the link observed between subclinical atherosclerosis and the presence of IRI reflects different consequences of a common phenomenon: the accelerated atherosclerotic process that is present in HIV-infected persons.

Both the proatherogenic effect of HIV itself through HIV-associated systemic inflammation and antiretroviral-induced metabolic disorders have been suggested to predispose to premature atherosclerosis.[37,38] Patients with IRI also showed a higher prevalence of traditional cardiovascular risk factors, in particular hypertrygliceridemia and diabetes.

Several studies have suggested a proatherogenic effect of HIV through HIV-associated systemic inflammation. HIV-infected patients with IRI tended to show higher levels of circulating cardiovascular biomarkers supporting the presence of a low-grade systemic inflammation than patients without IRI. In non–HIV-infected patients, a low-grade systemic inflammation has been associated with premature atherosclerosis[39] and the incidence of CVD events.[40]

The possible influence of antiretroviral treatment on IRI cannot be completely ruled out, given the relationship observed between cumulative use of antiretroviral drugs and renal impairment in the univariate analysis. Although we detected a relationship between cumulative use of antiretroviral therapy and the presence of IRI, the use of tenofovir, a generally well-tolerated drug that may cause tubular toxicity, was not statistically related. The limited number of patients in our series precludes us from drawing conclusions on the effect of individual drugs, but we consider that this hypothesis should be explored in greater depth.

TABLE 2. Differences in Renal Function Markers Between 145 Patients With and Without Subclinical Atherosclerosis

Renal function Marker	No Subclinical Atherosclerosis	Subclinical Atherosclerosis	*P*
N	52	93	—
Creatinine (mg/dL)	1.1 ± 0.2	1.2 ± 0.8	0.109
C-cystatin (mg/dL)	0.8 ± 0.2	0.8 ± 0.2	0.348
eGFR (mL/min/1.73 m^2)	96.8 ± 10.1	89.2 ± 10.7	<0.001
Annual decrease in eGFR, %*	1.3 (0.6–1.9)	1.5 (0.6–2.1)	0.366
ACR (mg/g)*	4 (3–7)	7 (4–16)	<0.001
Microalbuminuria, %	8.2	13.6	0.312

All values are expressed as mean ± SD unless otherwise specified.
*Expressed as median (IQR).
eGFR, glomerular filtration rate estimated by the CKD-EPI equation; annual decrease in eGFR, annual rate of decrease in eGFR over a period of 3 years; microalbuminuria, ACR >30 mg/g.

Clinical Implications

Management of HIV-infected patients to date has been based on optimization of 2 following surrogate parameters: viral load and the CD4 lymphocyte count. However, mortality data show that although HIV-infected persons are clearly living longer as a consequence of effective HAART, they may be dying earlier than the general population from conditions not traditionally associated with HIV infection, such as kidney disease and CVD.[41] Nowadays, one of the clinical challenges in the management of these individuals is the noninvasive detection of patients at increased risk of CVD to prevent the progression of atherosclerosis. We demonstrated that mildly decreased eGFR associated with a rapid decline in kidney function and/or albuminuria levels within the normal range was an important prognostic factor for increased cIMT. Although measurement of cIMT is considered the gold standard for the noninvasive detection of subclinical atherosclerosis, this approach cannot be generalized, as it is expensive and not universally available. Therefore, periodic monitoring of eGFR and ACR might help to better identify subjects at increased risk of CVD to initiate aggressive management of risk factors. Our findings provide support for the hypothesis that mild abnormalities of renal function can independently predict an increased atherosclerotic burden and behave as a useful surrogate marker of subclinical atherosclerosis.

Study Limitations

Our study is subject to a series of limitations. First, cross-sectional studies can neither prove causality nor distinguish between risk and prognostic factors for a disease, in this case, subclinical atherosclerosis. Second, we used a surrogate marker of cardiovascular events, namely, cIMT, instead of studying the incidence of coronary events. Nevertheless, cIMT is a validated surrogate marker of cardiovascular mortality and is the only test recommended by the American Heart Association for the assessment of the burden of atherosclerotic plaque.[18] Third, although we identified the presence of subclinical atherosclerosis with degrees of albumin excretion as low as 5 mg/g, further studies are needed to investigate the generalizability of our results to other HIV patient populations. In addition, ACR was measured only once in our study. Although it is unknown whether a confirmatory ACR determination should be performed when evaluating the presence of low-grade albuminuria, there is a potential risk of misclassification bias in our analysis, as urinary albumin excretion may provide false results under specific circumstances.[42] Fourth, given the sample size and the number of events of the dependent variable, we could not simultaneously adjust the effect for all the possible confounders, only for those variables producing a significant change in the crude odds ratio (>10%). However, we believe that the effect of the presence of IRI is properly adjusted, given the weakness of any possible residual confounders. Finally, most subjects in this cohort had IRI (64.1%), thus reflecting the poor specificity of definition "B". Consequently, our data must be interpreted carefully and should be reproduced in further studies in HIV-infected patients. It is also necessary to evaluate strategies to improve the diagnostic values of IRI before considering it a reliable marker of subclinical atherosclerosis when screening for CVD. In our opinion, these limitations are overwhelmed by the proof of concept that mild

TABLE 3. Diagnostic Values of the Four Definitions of Incipient Renal Impairment for the Diagnosis of Thicker Carotid Intima Media Thickness

	Incipient Renal Impairment	AUC	Sensitivity (%)	Specificity (%)	OR (95% CI)	*P*
"A"	eGFR 60–90 ± rapid decrease in eGFR	0.568	43.2	70.4	1.8 (0.8 to 3.7)	0.115
"B"	±ACR > 5 mg/g	0.645	84.1	44.9	4.3 (1.7 to 10.6)	0.001
"C"	±ACR > 10 mg/g	0.608	61.4	60.2	2.1 (1.2 to 4.9)	0.018
"D"	±ACR > 30 mg/g	0.582	50	66.3	1.9 (0.9 to 4)	0.067

AUC, area under the curve; eGFR, glomerular filtration rate by the CKD-EPI equation; eGFR 60–90, estimated glomerular filtration rate between 60 and 90 mL/min/1.73 m^2; rapid decrease in eGFR, decrease in eGFR >3% per year over a period of 3 years.

TABLE 4. Biomarkers of Inflammation and Vascular Damage in Patients With and Without Incipient Renal Impairment

	No Incipient Renal Impairment	Incipient Renal Impairment	*P*
N	52	93	—
CRP (mg/L)*	0.13 (0.05–0.30)	0.15 (0.07–0.43)	0.217
Interleukin-6 (pg/mL) *	1.9 (1.9–2.9)	1.9 (1.9–2.5)	0.876
Fibrinogen (mg/dL)	316.1 ± 73.7	328 ± 85.4	0.337
D-dimers (ng/mL)*	137 (87–254)	173 (72.5–378)	0.581
NT-proBNP (pg/mL)*	34.5 (19.3–60.5)	39 (22–106.5)	0.155

All values expressed as mean ± SD unless otherwise specified.
*Expressed as median (IQR).
NT-proBNP, N-terminal pro-B–type natriuretic peptide.

abnormalities of kidney function within the normal range may reflect an increased risk for CVD.

CONCLUSIONS

IRI is an independent predictor of increased cIMT (OR: 3.8; 95% CI: 1.3 to 11) and could be an easy and accessible way to identify patients with greater subclinical atherosclerosis and, therefore, an increased risk of CVD.

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ANEXO II: COMUNICACIONES

1- Talía Sainz, Laura Díaz, María Álvarez, María Luisa Navarro, María Isabel González-Tomé, María Isabel de José, José Tomás Ramos, Sergio Serrano-Villar, María Ángeles Muñoz y María José Mellado on behalf of the Cohort of HIV-infected Children of Madrid. **Increased subclinical atherosclerosis and immune activation in HIV-infected children and adolescents -the CaroVIH Study.** 19th Conference on Retrovirus and Opportunistic Infections, CROI, Seattle,Wa, March 2012. Oral Poster, Themed Discussion.

2- T. Sainz, M. Álvarez, M. L. Navarro, M.A. Muñoz y M.J. Mellado en representación de la Cohorte de Madrid de niños y adolescentes infectados por VIH. **Aterosclerosis carotidea como marcador de riesgo cardiovascular en niños y adolescentes infectados por VIH: estudio CaroVIH".** Premio a las mejores Comunicaciones Orales del Congreso Nacional de la Sociedad Española de Pediátrica (AEPED), Granada, Junio de 2012.

3- T. Sainz, S. Serrano-Villar, L. Díaz, MI. González-Tomé, MD. Gurbindo, MI. de José, MJ. Mellado, JT. Ramos, J. Zamora, S. Moreno, MA. Muñoz-Fernández. **The CD4/CD8 Ratio as a Marker of T-cell Activation/Exhaustion in HIV-Infected Children and Young Adults on Antiretroviral Therapy.** [Late-breaker abstract H-1570b]. 52nd International Conference on Antimicrobial Agents and Infectious Diseases (San Francisco). Washington DC, 2012

4- M. Álvarez Fuente, T. Sainz, C. Medrano, J.T. Ramos , D. Blazquez, M.I. De José, M.J. Mellado, M.L. Navarro, P. Rojo and M.A. Muñoz on behalf of the Madrid Cohort of HIV-infected children and adolescents, integrating the Pediatric branch of the National AIDS Research Network of Spain (CORISPE). **Evaluation of ventricular function in HIV-infected pediatric and adolescent patients: the use of Speckle tracking.** 20th Conference on Retrovirus and Opportunistic Infections, CROI, Atlanta, Ge, March 2013. Poster.

5- B. Jimenez, T. Sainz, L. Diaz, M.L. Navarro, M.I. Gonzalez-Tome, P. Rojo, M.J. Mellado, D.Gurbindo, J.T. Ramos, M.I. de Jose, M.A. Muñoz, Madrid Cohort of HIV-infected Children integrating the Pediatric branch of the National AIDS Research Network of Spain (CORISPE). **Low Bone Mineral Density in Vertically HIV-Infected Adolescents; Inflammation, Immune Activation and HIV-Related Factors**. 20th Conference on Retrovirus and Opportunistic Infections, CROI, Atlanta, Ge, March 2013. Poster.

6- T. Sainz, M. Santos-Sebastián, B. Jiménez, S. Jimenez de Ory, J. Saavedra, D. Blazquez, J.T. Ramos, M.J. Mellado, MI de José y M.L. Navarro on behalf of the Madrid Cohort of HIV-infected children and adolescents, integrating the Pediatric branch of the National AIDS Research Network of Spain (CORISPE). **Treatment simplification in HIV-infected children and adolescents**. 20th Conference on Retrovirus and Opportunistic Infections, CROI, Atlanta, Ge, March 2013. Poster

7- T. Sainz, M.Alvarez, ML. Navarro, J.T. Ramos, M.J. Mellado, MI. de Jose, D. Blazquez, J. Martinez, L Diaz, S. Serrano, M.A. Muñoz-Fernandez and P. Rojo, on behalf of the Madrid Cohort of HIV-infected children and adolescents, integrating the Pediatric branch of the National AIDS Research Network of Spain (CORISPE). **Ventricular function and cardiovascular risk in HIV-infected children and adolescents: the caroVIH study.** Oral poster. 31st Annual Meeting of the European Society for Paediatric Infectious Diseases, ESPID, Milan, Italy, May 28-June 1, 2013.

8- T. Sainz, A. Ortega, ML. Navarro, P. Rojo, J.T. Ramos, M.J. Mellado, S. Serrano, V. Estrada, D. Gomez-Garre and M.A. Muñoz-Fernandez on behalf of the Madrid Cohort of HIV-infected children and adolescents, integrating the Pediatric branch of the National AIDS Research Network of Spain (CORISPE). **Dysfunction of HDL particles in HIV-infected children and adolescents.** Oral poster. 31st Annual Meeting of the European Society for Paediatric Infectious Diseases, ESPID, Milan, Italy, May 28-June 1, 2013.

9- María Luisa Navarro, Talía Sáinz, Mª Isabel González-Tomé, Santiago Jiménez de Ory, Pere Soler-Palacín, María Espiau, Grupo de Trabajo CoRISpe. **Lost Opportunities: New Diagnoses of HIV-Infected Children In Spain** B**etween 2005-2011**. XII Congreso Nacional de la Sociedad española de Virología, Burgos, 9-12 Junio 2013. Comunicación oral.

Increased Subclinical Atherosclerosis And Immune Activation In HIV-Infected Children And Adolescents -The CaroVIH Study.

Talía Sainz, Laura Díaz, María Álvarez, María Luisa Navarro, María Isabel González-Tomé, María Isabel de José, José Tomás Ramos, Sergio Serrano-Villar, María Ángeles Muñoz y María José Mellado on behalf of the Cohort of HIV-infected Children of Madrid.

29th Conference on Retroviruses and Opportunistic Infections, CROI, Seattle, March 2012.
Themed Discussion, Oral.

Objectives. HIV patients present early cardiovascular disease (CVD). The study of subclinical atherosclerosis in subjects without CVD risk factors, such as children and adolescents, may help to clarify the specific influence of HIV infection on the atherogenic process. It is unknown if the recently described association between T-cell activation and senescence with carotid artery abnormalities is already present in childhood.

Methods. Carotid intima-media thickness (IMT) was measured in 122 HIV-infected children and young adults and 53 healthy controls matched by age, sex and body mass index (BMI). Markers of immune activation (CD38+HLADR+) and immune senescence (CD57+CD28-) were determined in a subgroup of 34 HIV patients and 11 controls.

Results. The mean age of the HIV-infected and uninfected subjects were 14.9 years (range 2.5 to 23.8) and 13.6 (range 2.9 to 22.6), respectively; smokers 15.5% vs 5.7%. Most HIV patients were female (64.8%), had undetectable viral load (76.4%), were vertically HIV-infected (96.7%) and all but 2 patients were on HAART.
IMT was thicker in HIV patients compared to uninfected subjects (0.434mm ± 0.025 vs 0.424 ± 0.018, respectively). After adjustment by age, sex, BMI and smoking status, HIV infection was independently associated with thicker IMT (odds ratio, 2.7; 95% confidence interval, 1.4-5.5; p=0.004). Among HIV-related variables, only a lower CD4 nadir was related to increased IMT (Spearman Rho = -0.18; p = 0.055).
Compared with HIV uninfected subjects, frequencies of activated T CD4+ cells were higher among HIV-infected children and young adults (p=0.002), with a border-line statistical significance for activated CD8+ cells (p=0.087) and senescent T CD4+ and T CD8+ cells. Viremic patients had the highest frequency of CD4+ and CD8+ activation and senescence, compared to patients with undetectable viral load and control subjects ($p<0.05$).
In the subgroup of viremic patients, those with increased IMT (above the median) showed a higher frequency of activated T CD4 cells (5.6 vs 2.3) and T CD8 cells (25.0 vs 19.0) that did not reach statistical significance.

*Conclusions***.** In our study in children and young adults, there was a three-fold increased risk of higher IMT due to HIV. The effect of the immunological status seems to be stronger than the effect of age in the early stages of life. Larger studies are warranted to evaluate the role of HIV-induced immune activation in the acceleration of atherogenesis since childhood.

Aterosclerosis carotidea como marcador de riesgo cardiovascular en niños y adolescentes infectados por VIH: Estudio CaroVIH

Talia Sainz Costa, M.ª Álvarez Fuente, M.ª Luisa Navarro Gómez, M.ª Ángeles Muñoz-Fernández, M.ª José Mellado Peña en representación de la *Cohorte de Madrid de seguimiento de niños y adolescentes infectados por VIH.*

61ª Congreso Nacional de la Asociación Española de Pediatría, AEPED, Granada 2012.
C145

Introducción: En un momento de preocupación creciente por el aumento de enfermedad cardiovascular en los sujetos infectados por VIH, el estudio de población pediátrica permite analizar el efecto de la propia infección, la activación y senescencia inmune y el tratamiento antirretroviral en la aceleración del proceso aterosclerótico, en ausencia de factores de riesgo cardiovascular clásicos.

Métodos: Estudio prospectivo de valoración de riesgo cardiovascular mediante medición del grosor íntima-media carotideo (IMT) en un grupo de niños infectados por VIH comparado con grupo control. Se recogieron además variables clínicas, antropométricas y analíticas. En un subgrupo de 34 pacientes y 11 controles se analizó activación y senescencia inmune mediante citometría.

Resultados: Se compararon 150 pacientes infectados (97% transmisión vertical, 97% en tratamiento, 76% carga viral indetectable) y 170 controles. La mediana de edad fue de 14,9±4,6 años y 14,4±4,6 respectivamente, 62% mujeres. No hubo diferencias entre ambos grupos en cuanto a edad, sexo, IMC, hábito tabáquico, tensión arterial o colesterol. Los pacientes con VIH presentaron perfil lipídico significativamente más aterogénico: HDLc (mg/dL) (49,8±12,4 vs 60,5±13,8); p <0,001), cociente colesterol total/HDLc (mg/dL) (3,7±1,1vs 2,8±0,8; p=0,000), y
triglicéridos (mg/dL) (107 [103-150] vs 68 [49,5-90]; p<0,001); y perfil glucémico basal también desfavorable; glucosa (mg/dL) (87±9,4 vs 82±10,4; p=0,001); índice HOMA (31,5 [21,6-61,7 vs 19,5[10,7-38,2]; p= 0,001).
Se observó un grosor significativamente mayor de IMT en los pacientes infectados frente a los controles (0,430mm±0,25 vs 0,420±0,22, p <0,001). Tras ajustar por sexo, edad, IMC y tabaquismo, la infección por VIH fue el único factor independiente asociado con una elevación de IMT por encima de la mediana (OR: 2,4, IC95%: 1,4-3,8; p <0,001). Los sujetos infectados presentaron mayor frecuencia de linfocitos CD4 activados (CD38+HLADR+) (p=0,002) y
de la frecuencia de linfocitos CD4 y CD8 senescentes (CD57+CD28-), aunque la diferencia no alcanzó la significación estadística. Los pacientes con cargas virales detectables presentaron mayor activación y senescencia (p<0,05) que los de carga viral indetectable y los controles,
especialmente aquéllos con IMT elevado (por encima de la mediana), aunque la diferencia no resultó estadísticamente significativa.

Conclusiones: La población de niños y adolescentes infectados VIH presenta un IMT discretamente superior al de controles sanos, lo que sugiere que el VIH es un factor de riesgo cardiovascular independiente, que ya ejerce su efecto desde edades tempranas de la vida. Los fenómenos de activación y senescencia inmune secundarios a la infección parecen participar junto a otros factores en este aumento de riesgo cardiovascular.

The CD4/CD8 Ratio as a Marker of T-cell Activation/Exhaustion in HIV-Infected Children and Young Adults on Antiretroviral Therapy

T. Sainz, S. Serrano-Villar, , L. Díaz, M. González-Tomé, M. Mellado, J. Ramos, MD, S. Moreno, M. Muñoz-Fernández.

52nd Interscience Conference on Antimicrobial Agents and Chemotherapy,ICAAC, San Francisco, September 9-12, 2012.
Poster. Late-Breaker Abstract. Reference: H-1570b

Background: An inverted CD4/CD8 ratio is a marker of immune senescence and a predictor of mortality in the general population and reflects oligoclonal expansions of senescent $CD8^+$ T cells. Herein we analyze the associations between the CD4/CD8 ratio and $CD8^+$ T cell activation, senescence and exhaustion in vertically HIV-infected children and adolescents on ART.

Methods: We performed a cross-sectional analysis in 37 vertically HIV-infected children and adolescents on stable triple ART. Laboratory measurements included T cell subpopulations, markers of $CD8^+$ T cell activation ($CD38^+HLADR^+$), senescence ($CD27^-CD58^+$), activation/exhaustion ($HLADR^+PD\text{-}1^+$) and exhaustion ($PD\text{-}1^+$).

Results: CD4/CD8 ratio showed a positive correlation with cumulative ART exposure ($r = 0.339$, $p = 0.040$), time on viral suppression ($r = 0.457$, $p = 0.004$), naïve $CD4^+$ T cells ($r = 0.336$, $p = 0.042$), naïve $CD8^+$ T cells ($r = 0.408$, $p = 0.013$), and a negative correlation with CD8+ T cell activation ($r = -0.414$, $p = 0.012$), exhaustion ($r = -0.389$, $p = 0.017$) and activation/exhaustion ($r = -0.752$, $p < 0.001$). After adjustment by age, $CD4^+$ T cell nadir, accumulated ART exposure and time with undetectable viral load, an inverted CD4/CD8 ratio independently predicted higher levels of $CD8^+$ T cells with an activation/exhaustion phenotype ($B = -0.033$, $p = 0.001$).

Conclusions: An inverted CD4/CD8 ratio may identify vertically HIV-infected children and adolescents with higher immune activation, senescence and exhaustion despite ART; a finding that may have implications both in diagnostic and therapeutic settings.

Evaluation Of Ventricular Function In HIV-Infected Pediatric And Adolescent Patients: The Use Of Speckle Tracking.

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30th Conference on Retroviruses and Opportunistic Infections, CROI, Atlanta, March 2013.
Poster.

Objectives. Previous studies have evidenced the presence of premature atherosclerosis and ventricular dysfunction in HIV-infected adults. Both the virus and prolonged medication might be partially responsible. Nowadays we have new echocardiographic techniques, like Speckle Tracking (ST), which analyzes ventricular torsion, that allow to diagnose early stages of ventricular dysfunction.

Methods. Multicentered study to evaluate ventricular function by echocardiography in HIV-infected children and adolescents, compared to a healthy control group matched by sex, age and body mass index (BMI). Clinical, anthropometric and analytical variables were recorded. Ventricular function was evaluated with a portable echo-device (Philips CX50) through: M-Mode (shortening fraction (SF) and ejection fraction (EF)), 2D, valvular Doppler, tissue Doppler and ST.

Results. 77 cases and 71 controls were included, median age was 15.2 years (IQR 10.04-18.19), 63.6% (49) females and BMI 19.4 (SD 3.59) No differences were observed in smoking habits. 96.2% were vertically HIV-infected, 78.2% had undetectable viral load, all but 4 patients were on HAART. Systolic function was lower in HIV+ (SF 36.3%(SD 6.41) and EF 66.2% (SD 8.39)) versus (SF 40.6% (SD 6.88) and EF 71.3% (SD 7.51)) ($p<0.001$), none of them had pathologic data (SF<28%, EF<50%).
No differences were found in diastolic function or in the tissue Doppler examination. Ventricular torsion was greater in HIV patients: 6,06° (SD 2.25) versus 5,49° (SD 1.97) ($p=0.09$).
Analyzing subgroups by age, we found a decreased diastolic function in adolescents over 13 years: ratio E/A doppler waves 1.76 in HIV+ versus 1.97 in HIV- ($p=0.037$), being these ratios non-pathologic. All other variables had no difference by age group.
In the HIV group, 5 patients presented systolic dysfunction, of which 2 had pathologic EF and SF. These patients had also higher viral load and worse immunologic status.

*Conclusions***.** Since childhood, HIV infected patients present a decrease in ventricular function in comparison to non infected patients. We observed an increase in the ventricular torsion in the pediatric HIV population independent of age and in accordance with a senescent myocardium.

Low Bone Mineral Density in Vertically HIV-Infected Adolescents; Inflammation, Immune Activation and HIV-Related Factors

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30th Conference on Retroviruses and Opportunistic Infections, CROI, Atlanta, March 2013.
Poster.

Background: International guidelines recommend ART initiation since birth for vertically HIV-infected children. However, few is known about the specific effects of treatment, HIV infection and immune activation on bone turn-over; a major concern for pediatricians, as alterations of bone mineral density during growth may have serious implications for our patients in the future.

Methods: Bone mineral density (BMD) was studied in a group of 24 HIV-infected children and adolescents. Osteopenia was defined as BMD< -1 z-score SD. Inflammatory markers (hsCRP, IL-6) and monocyte activation markers (sCD14) were determined, and T cell immune activation ($CD38^+HLADR^+$) and senescence ($CD27^-CD58^+$) were analyzed by flow cytometry.

Results: We included 24 vertically HIV-infected children and adolescents, all of them on ART, but only 70% virologically suppressed. 79.2% presented Vitamine D levels below the threshold of 30 ng/mL (54% below 20 ng/mL). Nine subjects met the criteria for osteopenia. Main characteristics of both groups are shown in the table.

Variable	Osteopenia	Normal BMD	p
Male (%)	44.4	7.1	0.056
Age (years)	18.1±2.6	14±4.3	0.007
BMI z-score (Kg/m²)	-1 [-1.7–0.0]	0.25 [-0.1–1]	0.01
CD4 nadir (cells/mL)	189 [86–208]	414 [268–709]	0.001
Months with CD4<200cell/mL	3.9 [0.5–6]	0 [0–0]	0.003
Years with detectable viral load	15.7 [13–17]	9 [4.8–15.1]	0.033
Cumulated ART exposure (years)	13.2 [7.6–16]	10.5 [7–14.7]	0.403
Cumulated PI exposure (years)	10.9 [6–13.7]	5.8 [0.5–10.7]	0.06
Vitamine D (ng/mL)	17.7 [12.6-26]	17.7 [13.6-22.2];	0.403
hsCRP (mg/dL)	0.28 [0–0.58]	0.17 [0.08–1]	0.557
IL-6 (ng/mL)	0.0 [0–0.8]	0.36 [0.0–2.4]	0.305
sCD14 (µg/mL)	1.6 [1.5–2.2]	1.6 [1.5–2.2]	0.600

Nine subjects had been exposed to TDF, but no association to BMD was detected. No association between low mineral density and immune activation/senescence could be found.

We explored the association between BMD and time with detectable viral load adjusting by a series of consecutive multivariate lineal regression analyses considering as potential confounders age, sex, BMI z-score, cumulated ART and PI exposure and CD4 nadir. Time with detectable viral load remained statistically significant (0.21, $p<0.001$), adjusted only by CD4 nadir.

Conclusions. A high prevalence of hypovitaminosis D and osteopenia was observed in this cohort of HIV-infected children and adolescents. Severe immune suppression and detectable viral loads during growth might be determinant factors for osteopenia.

Treatment Simplification In HIV-Infected Children And Adolescents

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30th Conference on Retroviruses and Opportunistic Infections, CROI, Atlanta, March 2013.
Poster.

Background: Achieving and maintaining good adherence to antiretroviral treatment (ART) is a challenge for clinicians treating HIV infected children and adolescents. Despite fear of tolerability issues and loss of virologic suppression, for this especial group of patients, treatment simplification strategies may improve adherence and decrease medication's adverse effects.

Methods: Retrospective analysis including immunovirologic and metabolic data following simplification of ART in patients integrating the Madrid Cohort of HIV-infected children and adolescents, between January 2001 and December 2011. We consider simplification every switch in ART regimen in a patient on virological suppression that resulted in reducing pill burden, dosing frequency or enhance tolerability.

Results: 122 episodes corresponding to 81 patients (55,5% females) were included. We observed a progressive increase in the number of simplifications during the study period; 56% corresponding to the last three years. Medium age at the moment of ART switch was 14 ±3.9 years, and median CD4 cell count 813/mm³ (627-1060). In a 90% of the cases, treatment change reduced pill burden, 35% decrease dosing frequency to once a day and 65% enhance tolerability. Fixed-dose combinations were used in a 54% of simplifications, mostly efavirenz, emtricitabine, and tenofovir disoproxil fumarate (43%). Medium follow-up time was 20 months (11-31), with loss of virologic suppression only in 5.7% of cases. There was an increase in CD4% at 12, 24 and 36 months of follow-up (all $p<0.05$). The increased in absolute number of CD4 lymphocytes did not reach statistical significance. Lipid profile was analyzed in those treatment simplifications that reduced metabolic toxicity; total cholesterol, triglycerides and low-density lipoprotein levels decreased along the study period (all $p<0.05$). However, high-density lipoprotein levels remain unchanged.

Conclusions: Treatment simplification strategies have proved to be safe and secure also in children and adolescents; most subjects maintain virologic suppressioon and there is an increased in CD4 suggesting adherence improvement. There is a need to implement strategies to reduce toxicity and improve adherence, convenience, and quality of life of our youngest patients on suppressive therapy.

Ventricular Function and Cardiovascular Risk In HIV-Infected Children And Adolescents; The CaroVIH Study

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31st Annual Meeting of the European Society of Pediatric Infectious Diseases, ESPID, Milan May 28-June 1, 2013.Oral poster

Background. Previous studies have evidenced the presence of premature atherosclerosis and ventricular dysfunction in HIV-infected patients. Ultrasound measurement of carotid intima-media thickness (IMT) and *Speckle Tracking Echocardiography (STE)* may serve as early markers of cardiovascular disease.

Methods. Clinical, anthropometric and immunovirological variables were analyzed in a cohort of vertically HIV-infected children and adolescents and age-and-sex matched controls. Inflammatory and cardiovascular biomarkers as well as markers of immune activation and senescence were determined, and a complete echocardiographic study was performed, including IMT, shortening and ejection fraction (SF, EF), tissue Doppler and *STE*.

Results. 150 HIV-infected subjects and 150 controls were included. Mean age was 14.8±4.9 years, 62% were female. Most HIV patients had undetectable viral load (76.4%), were vertically HIV-infected (96.7%) and all but 2 patients were on ART. HIV-infected subjects showed thicker IMT compared to uninfected subjects (0.434mm ± 0.025 vs 0.424 ± 0.018, respectively), and lower systolic function (p<0,001), although values were within normal ranges. Ventricular torsion was greater in this group: 6,06° (SD 2,25) vs 5,49° (SD 1,97) (p=0,09). Among cardiac biomarkers, only tPA and VCAM were higher in HIV+ subjects compared to controls (all p<0.05), and there was a non-significant trend to higher hsCRP. HIV-infected subjects presented higher frequencies of activated CD4 T-cells (p=0.016), and non-suppressed patients showed also higher frequencies of senescent CD8 T-cells (p<0.001). No relation to cardiac markers could be stablished.

Conclusions. Subclinical atherosclerosis and early signs of senescence of the myocardium are already present in vertically HIV-infected children, suggesting that despite ART cardiovascular risk is increased in these patients and should be carefully monitored since childhood.

Dysfunction of HDL Particles In HIV-Infected Children And Adolescents

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31st Annual Meeting of the European Society of Pediatric Infectious Diseases, ESPID, Milan May 28-June 1, 2013.Oral poster

Background. We hypothesized that pro-atherogenic mechanisms related to immune activation during HIV-infection may impair HDL functionality, increasing cardiovascular risk in HIV-infected subjects.

Methods. Plasma was obtained from healthy controls and vertically HIV-infected children on ART. HDL were isolated and tested in vitro for its ability to inhibit the chemotaxis of monocytes (THP-1 cells), assessed by Transwell® cell culture chambers, in the presence of monocyte chemoattractant protein (MCP-1). T-cell activation (HLADR+CD38) was measured by flow-cytometry.

Results. We included 14 HIV-infected subjects and 5 healthy controls; 13 (65%) were female, and the mean age was 16.3±2.2 years. All patients were on ART (7 in a LPV/r containing regimen, 3 ATZ/r and 2 EFV), but only 9 had achieved virological suppression. Median CD4 cell count was 841 cells [498-1126], CD4% 31[25.7-38.1] and CD4 nadir 371 cells [160-595]. HIV-infected subjects showed a slightly worse lipid profile, although the differences were not statistically significant. HDL anti-inflammatory function was decreased in HIV-infected subjects (p=0.494), and especially on those with detectable viral load, as shown in the graph *A* (all p >0.05). No association to ART regimen was found, but there was a significative correlation between inhibition and CD4 nadir (Spearman Rho= 0.547, p=0.043, graph *B*) and immune activation (Spearman Rho= -0.407, p=0.083, graph *C*).

Conclusions. Our observations suggest that the anti-inflammatory properties of HDL particles are defective in HIV-infected children. Successful ART only partially reverse this situation. To explore new strategies to increase HDL functionality rather than HDL levels might be of interest in HIV infection.

Lost Opportunities: New Diagnoses of HIV-Infected Children In Spain Between 2005-2011.

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XII Congreso Nacional de la Sociedad española de Virología, Burgos, 9-12 Junio 2013. Comunicación oral.

Background: Public health initiatives aiming to prevent vertical transmission of HIV infection led in the last few years to a dramatic decline in the incidence of HIV infection among children in high-income countries. The objective of this study is to analyze the main characteristics of the few pediatric cases diagnosed in Spain in the last seven years.

Methods: Cross-sectional study including new diagnoses of HIV infection between January 2005 and December 2011. Demographic, clinical and inmunovirologic data were collected retrospectively from the CORISPE Database, which includes all children living with HIV (CLHIV) in Spain.

Results: The Spanish Cohort of HIV-infected Children (CoRISpe) includes to date 925 CLHIV, of whom 171 were diagnosed during the study period (51.7% female) and 86% of cases corresponded to vertical transmissions.E1.7% were born to a eighty-seven children (59%) were born in Spain, of them 51.7%were born to a Spanish mother, followed by 21.8%) born to a sub-Saharan mother. Regarding children born in Spain, data about perinatal conditions were available for 77 patients: 29.9% of mothers were diagnosed before pregnancy, 23.4% during pregnancy, 10.4% during labour and 36.4% after delivery.

Median age of the children born in Spain at the moment of diagnosis was 0.4 years (IQR: 0.1-1.6), while it was 3.3 years (IQR: 2-6.4) for children born abroad ($p<0.001$). Median CD4/mm^3 was 1403 (IQR: 691-2749) and 840 CD4/mm^3 (IQR: 432-1274) respectively, and CD4%: 27 (IQR: 19-45) and 20 (IQR: 16-27.8), respectively (all $p<0.001$).

Conclusions: Despite widespread routine testing and the efficacy of specific interventions to avoid mother-to-child transmission, there are ongoing new diagnoses of HIV infection among children in our country. CLHIV born abroad are diagnosed late, and accordingly show a worse immunological condition. Up to 59,2% of the new diagnoses performed during the study period correspond to children born in Spain, representing lost opportunities to avoid HIV infection, mainly due to delayed maternal diagnosis or failure to implement prophylaxis of vertical transmission.

ANEXO III:

CONSENSO DE MANNHEIM

CONSENSO DE MANNHEIM PARA LA MEDICIÓN DEL GROSOR INTIMA-MEDIO CAROTIDEO.

A continuación se resumen las recomendaciones del Consenso de Mannheim recogidas por Toulboul et al. en su artículo publicado en 2004 y actualizado en 2007 en Cerebrovascular Diseases, para la medición del grosor íntima-medio carotideo.

Definiciones:

- GIM: Patrón de doble línea visualizado por ecografía en ambas paredes de la arteria carótida común, en un corte longitudinal. Formado por dos líneas paralelas que corresponden a la capas íntima y media-adventicia.
- Placa: Estructura focal que protruye hacia la luz arterial, con un grosor de al menos 1,5mm, o que protruye 0,5mm o el equivalente al 50% de GIM de las regiones contiguas.

Sujetos a estudio:

Participantes en ensayos clínicos y epidemiológicos relacionados con las enfermedades vasculares, al igual que se recogen otros factores de riesgo cardiovascular.

Lugar de medición:

Se elegirá un lugar libre de placa para la medición del GIM, a nivel de la arteria carótida común, del bulbo carotideo o en el origen de la arteria carótida interna. Si se realizan mediciones a nivel de diferentes localizaciones, los datos se analizarán por separado.

Procedimiento:

Para la medición del GIM se requieren un equipo de ultrasonografía en modo B de alta resolución, con una sonda lineal de al menos 7MHz. Se obtendrá un aimagen longitudinal del vaso, estrictamente perpendicular a la sonda. La medición se realizará en la pared posterior del segmento arterial elegido, que deberá tener al menos 10mm de longitud. Es necesario adquirir imágenes de alta calidad para asegurar una medición fiable. Se recomienda emplear sistemas automáticos de medición, que realizan una media de 150 determinaciones en un tiempo muy breve. Se obtendrán simultáneamente los diámetros arterial y luminal correspondiente al segmento medido.

Deberá evaluarse el coeficiente de correlación intraclase para determinar la variabilidad inter e intra-observador, tanto para la medición del GIM como para la determinación de la presencia de placa.

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