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TESI DOCTORAL

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dirigida per Dr Jordi Camps i Andreu

Departament de Ciències Mèdiques Bàsiques

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CERTIFICO:

Que la present Tesi Doctoral titulada "Influència de la Paraoxonasa-1 (PON1) en l'evolució de la infecció pel virus de la Immunodeficiència Humana-1 i les seves complicacions metabòliques", ha estat realitzada sota la meva direcció al Centre de Recerca Biomédica, i que compleix els requeriments necessaris per optar al títol de Doctor.

Per a que així consti, i tingui els efectes oportuns, signo aquest certificat.

Reus, 10 de desembre de 20

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Agraïments

Aquesta tesi està dedicada a tots els pacients afectats pel VIH de l'hospital de Reus. Cada un de vosaltres heu deixat un record que m'acompanyarà i em motivarà sempre per seguir treballant i lluitant.

M'agradaria agraïr molt especialment, al director d'aquesta tesi, el Dr. Jordi Camps, tot el suport i ajuda que m'ha ofert. És gràcies al seu compromís que he pogut arribar a finalitzar i presentar aquesta tesi. Ha estat la persona que ha assumit el paper de director des del principi fins al final, tot i les dificultats. Sempre m'has donat suport, has estat imparcial, just i molt pacient. Gràcies Jordi, per donar-me l'oportunitat d'acabar el meu treball i per tot el que m'has ensenyat i demostrat. Espero que el "browny" hagi valgut la pena i també espero arribar a ser una ponòloga digne de formar part d'un grup tant important com el teu. Gràcies per tot, "amigo".

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Gràcies, Asun, per ser un gran "puntal". Has sigut la meva "jefa" i la meva "evaluadora"en més d'alguna ocasió. Per mi has sigut un exemple a seguir, ets l'objectivitat, professionalitat i eficàcia personalitzada... els pacients saben que sempre hi ets ... i quants secrets professionals hem compartit! Gràcies.

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A la gent de la URL, Merche, Ana (a veure si passem visita juntes!), Gemma, Marta... sempre disposats a col·laborar i treballar.Gràcies.

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Índex d'abreviatures

ABCA1	ATP-binding cassette transporter ABCA1
ADN	Àcid desoxiribonucleic
ALT	Alanina aminotransferasa
Apo A-I	Apolipoproteina A-I
Apo A-II	Apolipoproteina A-II
ApoE	Apolipoproteïna E
Arg	Arginina
AST	Aspartat aminotransferasa
BCA	Bulb Carotid Artery
CAD	Coronary artery disease
CCA	Common Carotid Artery
CVD	Cardiovascular disease
ECV	Esdeveniments cardiovasculars
ELISA	Enzime-Linked Immunosorbent Assay
FAL	Fosfatasa Alcalina
FRC	Factors de risc cardiovascular
GGT	g-glutamiltranspeptidasa
GIM	Gruix íntima mitja
Gln	Glutamina
HDL	High Density Lipoprotein
ICA	Internal Carotid Artery
IMC	Índex de massa corporal
IP	Inibidors de la proteasa
ITIAN	Inhibidors de la transcriptasa inversa anàlegs de nuceòtids
ITINAN	Inhibidors de la transcriptasa inversa no anàlegs de nucleòtids
KO	Knockout
LDL	Low Density Lipoprotein
Leu	Leucina
MCP-1	Monocite Chemotactic Protein-1
MCV	Malalties cardiovasculars
Met	Metionina
mRNA	Àcid ribonucleic missatger
OR	Odds Ratio
PCR	Polimerase Chain Reaction
PON1	Enzim paraoxonasa. Producte del gen PON1
PON1	Gen que codifica PON1
PON2	Enzim paraoxonasa. Producte del gen PON2
PON2	Gen que codifica PON2
PON3	Enzim paraoxonasa. Producte del gen PON3
PON3	Gen que codifica PON3
ROS	Reactive Oxygen Species
SIDA	Síndrome de la Immunodeficiència Adquirida
SNP	Single Nucleotide Polymorphism

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TAR	Tractament antirretroviral
TAD	Tensió arterial distòlica
TAS	Tensió arterial sistólica
VIH-1	Virus de la immunodeficiència humana tipus 1

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JUSTIFICACIÓ

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Justificació

La infecció pel VIH ha esdevingut una malaltia crònica gràcies a la introducció del tractament antiretroviral d'alta activitat (1). Aquesta infecció afecta actualment a 33.2 milions de persones arreu del món. Tot i la disminució de la mortalitat, la incidència de complicacions metabòliques relacionades amb el tractament i la infecció adquireixen cada cop més rellevància clínica afectant a la morbimortalitat dels pacients (2-4).

La infecció pel virus del VIH promou un estat pro-oxidatiu que afavoreix la mateixa replicació viral i l'aparició d'alteracions metabòliques com l'aterosclerosi o la lipodistròfia (5-7). L'aterosclerosi és reconeguda com una malaltia inflamatoria crònica iniciada per l'oxidació de les lipoproteïnes a l'espai subendotelial (8). La paraoxonasa-1 (PON1) és un enzim associat a les lipoproteïnes d'alta densitat (HDL) que li confereix gran capacitat antioxidant en prevenir l'oxidació de les lipoproteïnes (9,10). Tot i que el seu sustrat fisiològic encara no es coneix, s'ha estudiat el seu rol protector en diverses malaties cròniques com la diabetis (11), l'artritis reumatoide (12), el lupus eritematós sistèmic (13) i diverses hepatopaties (14). Per aquesta raó creiem interessant investigar la possible funció d'aquest enzim antioxidant en la evolució immunològica i virològica de la infecció pel VIH i així també en l'aparició de complicacions metabòliques com l'aterosclerosi i els esdeveniments cardiovasculars d'aquests pacients.

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INTRODUCCIÓ

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1. Paraoxonases

Es coneix com paraoxonases (PON) els productes proteics de tres gens anomenats *PON1, PON2 i PON3* (15) localitzats, en els humans, en posicions adjacents al cromosoma 7 (7q21.3-q22.1). Aquests tres gens comparteixen una homologia estructural d'un 70% pel que es pensa que provenen d'un precursor gènic comú (16,17).

En humans els gens de *PON1* i *PON3* s'expressen sobretot al fetge (18) i també al ronyó. L'expressió de *PON2* es considera ubíquota (19). En sang perifèrica els enzims PON1 i PON3 es troben units a les lipoproteïnes d'alta densitat (HDL) i en canvi, el producte proteic PON2 es troba només a nivell intracel·lular (20,21).

Dels tres enzims, el més estudiat fins ara, ha estat PON1. L'enzim PON1 es coneix amb el nom de *paraoxonasa* per la seva capacitat per hidrolitzar l'insecticida paratió, tot i que també se'l pot anomenar *arilesterasa* en funció del substrat que s'utilitzi per a la seva determinació (22,23).

Les funcions fisiològiques dels productes gènics de PON2 i PON3 no són encara conegudes. S'han associat dos polimorfismes del gen de *PON2* a la regió codificant, $PON2_{148}$ (A/G) i $PON2_{311}$, amb un augment del risc de malaltia cardiovascular (S/C) (24,25). S'ha observat que PON2 té capacitat per protegir les partícules de LDL de l'oxidació tot i no trobar-se unida a les HDL i també té capacitat per hidrolitzar les lactones però no el paraoxó o altres xenobiòtics com PON1 (26, 27). PON3 és el membre més recentment identificat i donat que s'expressa en menor quantitat, es pensa que la seva importància és inferior (28).

2. L'enzim paraoxonasa-1

Mazur, l'any 1946, va descriure per primera vegada la degradació enzimàtica de compostos organofosforats en teixits, com el fenilacetat (activitat arilesterasa) o el paraoxó (activitat paraoxonasa) (29). Posteriorment Playfer i cols. (30) van descobrir que la variabilitat en l'activitat paraoxonasa era considerable identificant diferents variants gèniques localitzades en un mateix

locus autosòmic. Es diferenciaven en funció de les activitats paraoxonases/arilesterases dos o tres fenotips corresponents a activitats baixes, altes i mitjanes en sèrum. Posteriorment Eckerson i cols. (31,32) van estudiar aquestes tres variants fenotípiques. A les variants amb baixa activitat els va anomenat AA, AB a les intermèdies i BB a les altes. Adkins i cols. Una dècada després van identificar dues posicions polimòrfiques a la regió codificant de *PON1*: Arg/Gln a la posició 192 (polimorfisme $PON1_{192}$, amb dos al·lels anomenats Q i R que es corresponen a les variants fenotípiques A i B respectivament) (33); i Leu/Met a la posició 55 (polimorfisme $PON1_{55}$, amb dos al·lels anomenats L i M) (34).

És a dir, utilitzant com a substrat el paraoxó, el polimorfisme de *PON1* a la posició 192 amb la isoforma Q (substitució d'una arginina per una glutamina) correspon al fenotip A amb activitats baixes i la isoforma R (substituint una glutamina per una arginina) correspon amb el fenotip B amb activitats més altes, tal i com es demostra a la **Figura 1**.

En quant al polimorfisme $PON1_{55}$ no s'ha establert una associació tan clara amb l'activitat i la concentració de l'enzim, tot i que sembla que la isoforma M està associada a una activitat més baixa (35).

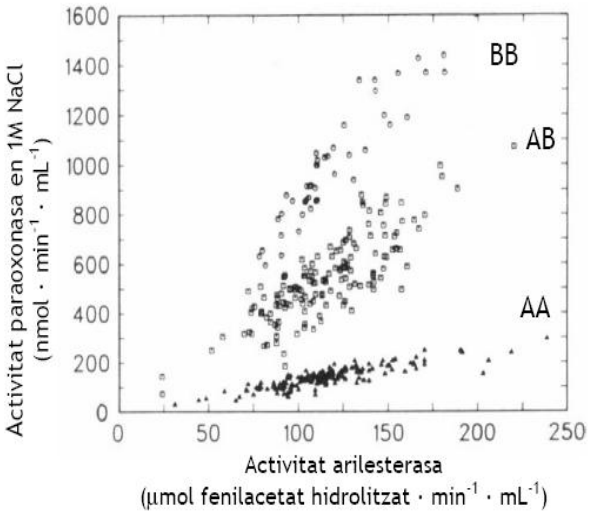


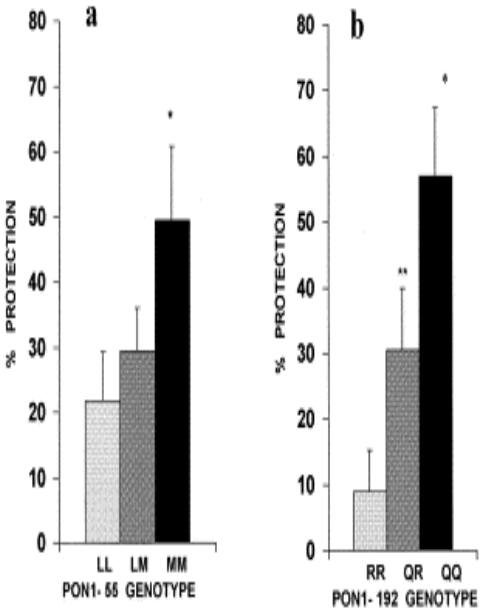
Figura 1. Representació gràfica de l'activitat paraoxonasa estimulada amb sals vs l'arilesterasa. S'observen clarament els 3 polimorfismes AA, AB i BB (31).

2.1. Funció fisiològica de PON1

2.1.1. Degradació de peròxids lipídics

La capacitat d'hidrolitzar els organofosforats per part de PON1 previndria la intoxicació a nivell del sistema nerviós central per aquests agents tòxics (36), d'altra banda, és important considerar que no són presents habitualment en la sang humana. Aquesta premissa i el fet que la especificitat de substrat fisiològic de PON1 no ha estat caracteritzada completament, ha provocat el debat sobre quina podria ser la funció fisiològica de PON1.

No va ser fins a la dècada dels 90 en que el grup de Mackness et al. van descobrir que PON1 unida a les HDL i també purificada era capaç d'inhibir la formació de lipoperòxids durant el procés d'oxidació de les lipoproteïnes de baixa densitat (LDL) *in vitro* (37,38).



Les variants gèniques de les posicions *PON1*$_{192}$ portadors de l'al·lel Q i a la posició *PON1*$_{55}$ els portadors de l'al·lel M, serien els al·lels que conferien més estabilitat enzimàtica i més capacitat per protegir davant l'oxidació dels lipoperòxids (39). (**Figura 2**)

Figura 2. Efecte dels polimorfismes de *PON1*$_{55}$ (a) i *PON1*$_{192}$ b) i l'habilitat de les HDL de protegir contra la modificació oxidativa (39).

Altres treballs de Mackness també demostraven que aquesta capacitat antioxidant de l'enzim estava determinada pel genotip de *PON1.*

En la **figura 3** es resumeixen, en un esquema, les posicions de les principals variants gèniques dels gens de *PON1, PON2, PON3*. Les seves associacions a l'activitat de l'enzim i l'associació a diferents malalties també ha sigut objectiu d'altres estudis.

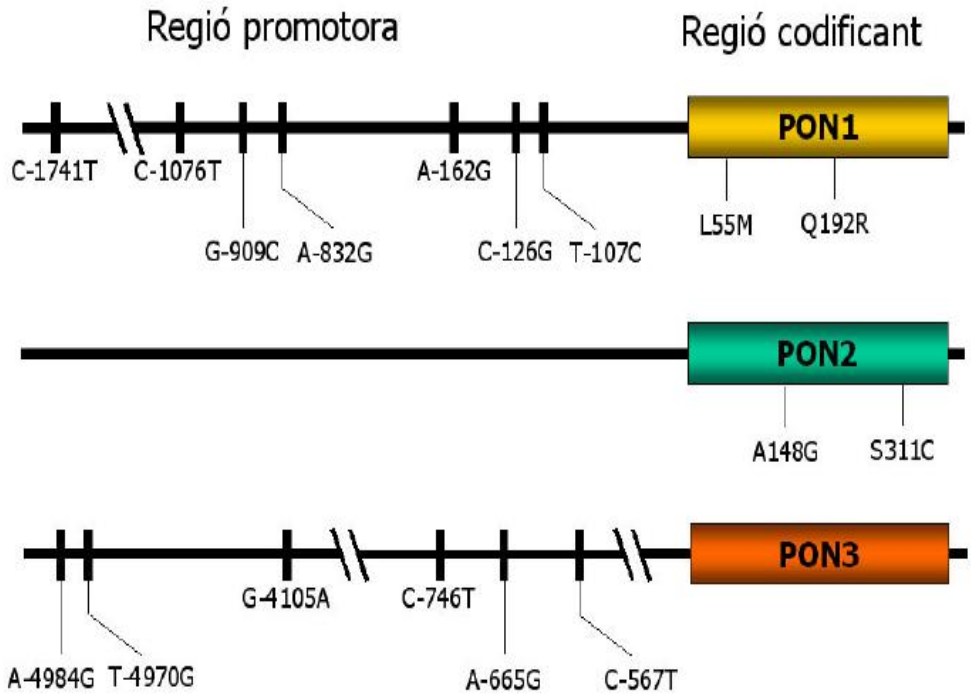


Figura 3. Representació esquemàtica dels polimorfismes més prevalents a la societat per *PON1, PON2 i PON3* (15,16).

Posteriorment aquests resultats es van confirmar per altres grups (39,40), destacant el d'Aviram i col·laboradors (41) amb diverses publicacions que confirmaven la protecció conferida per PON1 degradant colesteril-ésters i fosfolípids oxidats continguts a les lipoproteïnes oxidades. Així mateix, PON1 també afavoreix el transport revers de colesterol via ABCA1 (42). És a dir, PON1 seria un enzim antioxidant i amb funcions "protectores" en quant al procés arterioscleròtic.

Cal destacar també que el mecanisme d'hidròlisi dels lipoperòxids és diferent al del paraoxó, ja que el grup sulfhidril lliure de la cisteïna 284 és necessari per la

protecció contra la peroxidació lipídica de les lipoproteïnes i no es requereix per la hidròlisi del paraoxó (43).

Els estudis iniciats per Shi i cols. basats en models animals confirmen aquestes funcions *in vivo* (44) gràcies a la creació de ratolins *Knockout* (KO) per PON1 (*PON1*$^{-/-}$) i ratolins transgènics per PON1 humana (*hPON1Tg*) (45). El ratolí doble KO pel *PON1*$^{-/-}$ i apolipoproteïna E *(apoE*$^{-/-}$*)* presentava un augment de l'oxidació lipídica i de lesions arterioscleròtiques respecte el ratolí *apoE*$^{-/-}$. Les HDL aïllades del ratolí *PON1*$^{-/-}$ eren incapaces per prevenir l'oxidació de la LDL. D'altra banda, la sobre-expressió de PON1 humana en ratolins inhibeix la formació de peròxids lipídics en l'HDL i protegeix l'estructura i la funció de la LDL (46-50).

2.1.2. Estructura i activitat nativa de PON1

PON1 és un enzim amb capacitat per hidrolitzar múltiples sustrats, tal i com hem descrit prèviament (51,52). Fins i tot, PON1 té capacitat per hidrolitzar certs fàrmacs, com els èsters d'estrògens, les estatines o la warfarina (53-55). Per aquesta raó, PON1 ha estat inclosa en aquets grup d'enzims d'ampli espectre o també anomenats "enzims promiscus" (56,57). Aquesta diversitat de substrats, es pensa, que és resultat de l'evolució enzimàtica que es trobava als organismes més primitius, com els invertebrats. El disposar d'uns "enzims ancestrals" amb múltiples funcions catalítiques permetia mantenir la homeòstasi d'aquests organismes primitius (58,59).

Una interessant línea de recerca impulsada pel grup de Tawfik i cols. ha estat centrada en investigar quina ha sigut la funció nativa i l'evolució enzimològica de les paraoxonases (60,61).

La dificultat en poder purificar PON1 per poder estudiar l'estructura i el seu mecanisme d'acció ha estat un dels inconvenients per tal de descobrir quin és el substrat nadiu. A partir de l'aplicació de metodologia d'evolució dirigida (62), s'ha formulat la hipòtesi que PON1 s'estructura en forma de turbina (*β-propeller*) de sis fulles amb un centre actiu implicat en l'anclatge a les HDL (**Figura 4**). Al centre del túnel es troben dues molècules de calci. El calci inferior, és probablement estructural, mentre que el calci superior se'l considera

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catalític (63). Les tres hèlix –α, localitzades a la part superior de la turbina, estan involucrades en l'anclatge de PON1 a la partícula d'HDL. Dues de les tres hèlix es disposen deixant "tancat" el centre actiu (64).

Aquest mateix grup, mitjançant estudis d'evolució dirigida, juntament amb estudis d'estructura-funció, van establir que la funció primordial de PON1 és ser un enzim amb activitat lipolactonasa (65-67).

L'estudi de Harel i cols. també proposa un mecanisme catalític de PON1, basat en la parella d'histidines His^{115}-His^{134}, totes dues molt properes al calci catalític (68). Recentment s'ha demostrat que aquesta parella d'histidines és necessària tant per l'activitat lactonasa de PON1 com per la degradació de lipoperòxids (69).

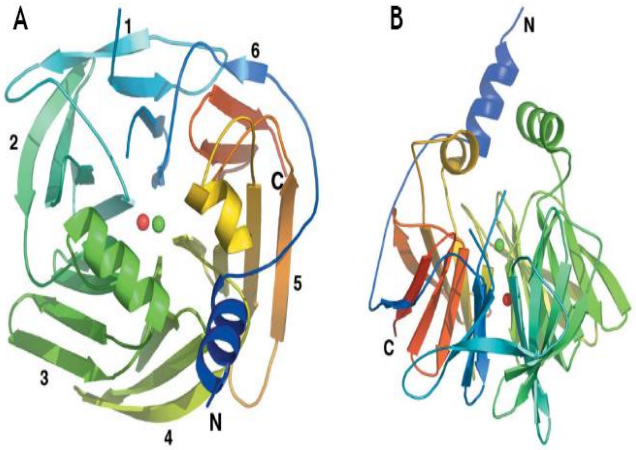


Figura 4. Estructura de PON1. A: vista des de dalt de la turbina amb les seves 6 fulles. Al centre es representen com a dues esferes els dos àtoms de calci. B: vista de la turbina de costat. En ambdues figures es representa coma N l'extrem N-terminal i C el C-terminal (63).

2.2. Síntesi i determinants de l'activitat paraoxonasa en sèrum

La síntesi de PON1 és principalment hepàtica tal i com han demostrat treballs previs (70). Donada la relació entre les hepatopaties i l'estrès oxidatiu s'han realitzat diversos estudis sobre la funció que pot desenvolupar PON1 en l'evolució d'aquestes malalties i la progressió a cirrosi hepàtica (71,72).

Ferré i col. van demostrar que en una cohort de malalts amb hepatopatia crònica de diferents etiologies l'activitat front el paraoxó de PON1 discriminava entre pacients i individus sans. Afegint l'activitat paraoxonasa a la bateria estàndard d'estudis bioquímics de funció hepàtica, la determinació de l'activitat

paraoxonasa augmentava la sensibilitat diagnòstica sense disminuir-ne l'especificitat (73-77).

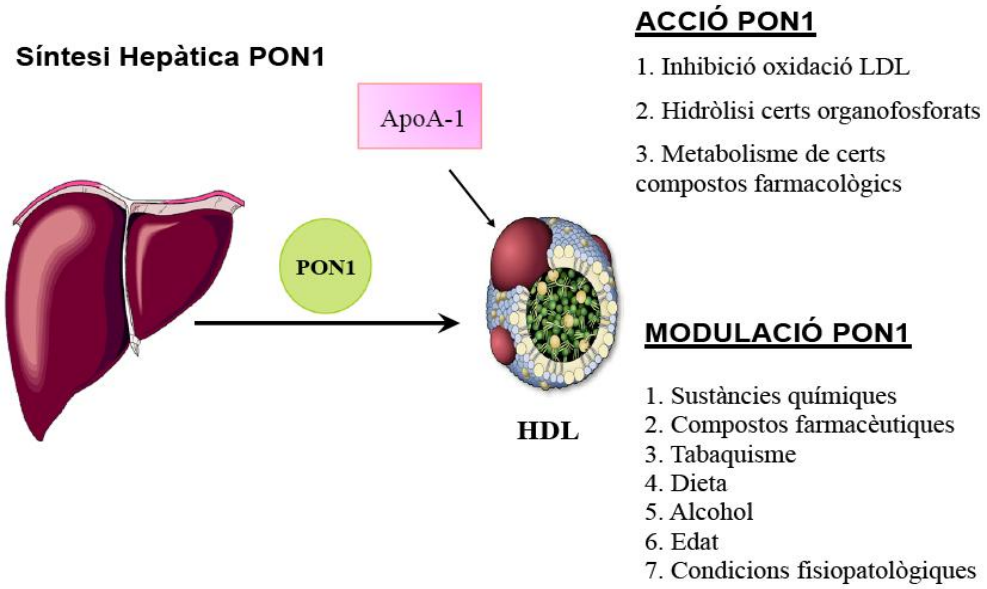
Així doncs, com hem esmentat prèviament, l'activitat de PON1 està influenciada pels polimorfismes de PON1 principalment a les posicions 192 i 55 però també es veuria afectada per malalties hepàtiques (78-81).

A més, darrerament, l'estudi de les paraoxonases en diverses malalties que promouen un increment de l'estrès oxidatiu ha motivat un increment exponencial en el nombre de publicacions en la darrera dècada (82-89). S'han publicat estudis de la possible funció de les paraoxonases en diverses malalties com són la diabetis, malalties neurològiques com l'Alzheimer i el Parkinson, el lupus eritematós sistèmic i l'artritis reumatoide (90-92).

L' activitat sèrica de l'enzim també es veu influenciada per altres factors ambientals com són factors dietètics (93-95), consum d'alcohol (96), consum d'antioxidants com el suc de magrana (97), el consum de tabac (98,99) i diversos fàrmacs com les estatines i els estròGens (53,54).

Aquesta possibilitat de modular l'activitat de PON1 fa encara més interessant l'estudi de les paraoxonases ja que podria ser considerada com un objectiu terapèutic o farmacològic en aquelles malalties on l'estrès oxidatiu i l'oxidació dels lipoperòxids poden tenir repercussions clíniques .

Figura 5: Efectes biològics i modulació de PON1 (93).



2.3. PON1 i inflamació

La principal font de colesterol extracel·lular prové de les LDL. Al ser oxidades les LDL produeixen una sèrie de fosfolípids biològicament actius que són mediadors dels esdeveniments inflamatoris que es presenten durant el desenvolupament de la placa d'ateroma (104). Els fosfolípids oxidats de les LDL com el 1-palmitoil-2-(5-oxovaleroil)-*sn*-glicero-3-fosforilcolina (POVPC) i el 1-palmitoil-2-glutaroil-*sn*-glicero-3-fosforilcolina (PGPC) afavoreixen l'adhesió dels monòcits a les cèl·lules endotelials i la transmigració de monòcits a l'espai subendotelial a través de l'estimulació de la producció de la quimioquina atraient de monòcits (MCP-1) (105,106).

Com ja és conegut, les HDL són partícules que tenen un paper ben reconegut en quan a la seva capacitat antiaterogènica degut a la seva habilitat per extreure el colesterol dels teixits perifèrics a partir de la interacció del receptor ABCA1 i la ApoA1 (107). Però a més d'aquesta funció, les partícules HDL també intervenen en la modulació de la resposta inflamatòria induïda pels fosfolípids oxidats de les LDL. Les HDL inhibeixen aquest estímul inflamatori a partir de la inhibició de l'oxidació dels fosfolípids mitjançant els enzims antioxidants associats a les HDL (108) (**Figura 6**).

D'aquests enzims antioxidants destaquem PON1 tot i que hi ha tres enzims més amb reconeguda capacitat per inhibir la oxidació de les LDL com són el Platelet-Activating Factor Acetylhydrolase (PAF-AH), lecitina:colesterol aciltransferasa (LCAT) i la glutation selenoperoxidasa (GS) (106, 111,112).

Mackness i cols. van demostrar in vitro la funció antiinflamatòria directa de PON1 (113,114). Al cultivar cèl·lules endotelials incubades amb LDL oxidada van demostrar que PON1 recombinant i PON1 unida a les HDL inhibia la producció de MCP-1 a partir de la inhibició de la producció de les LDL oxidades, això no succeïa a l'incubar les cèl·lules amb HDL d'origen aviar que no conté PON1 (115).

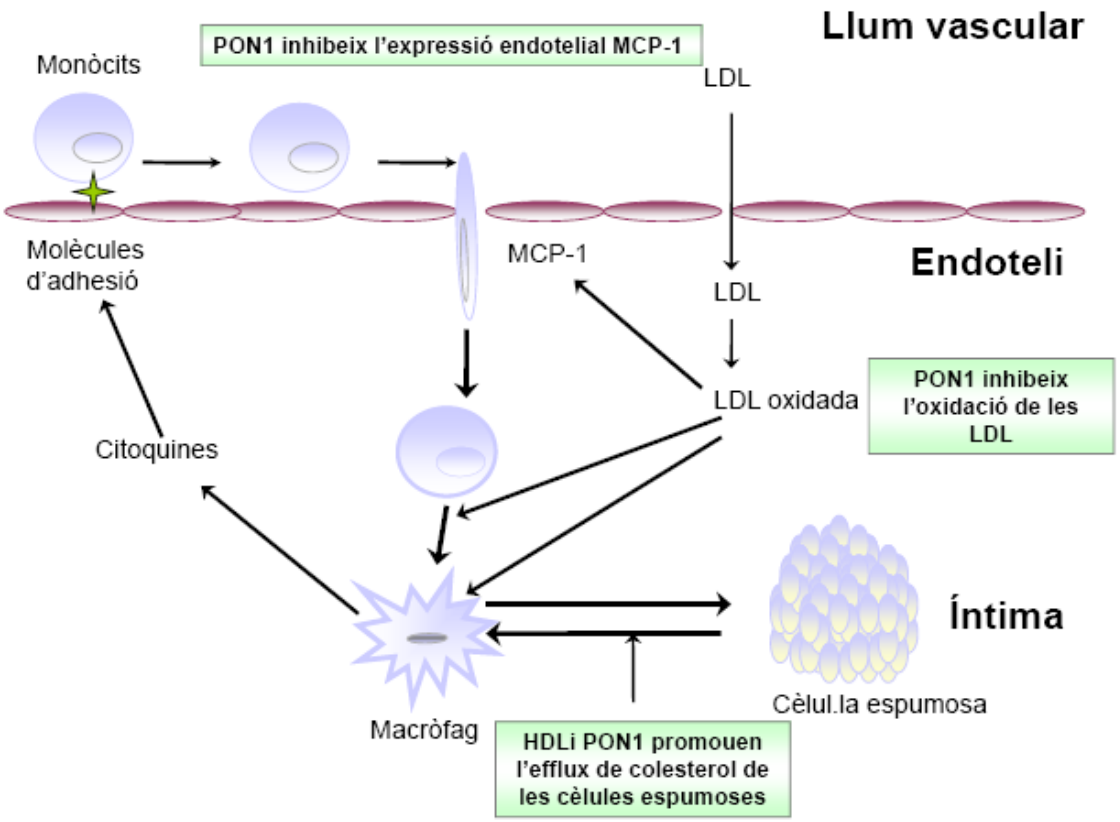


Figura 6. Esquema del paper protector de PON1 en les fases inicials de l'arteriosclerosi. Adaptada de Camps i cols. (109) i Navab (110).

La composició de les lipoproteïnes i enzims antioxidants associats a l'HDL es modifica en resposta a estímuls pro-inflamatoris o reactants de fase aguda. Tal i com hem descrit l'HDL és antiinflamatòria en estat basal (116), però sota estímuls que promouen una resposta de fase aguda les partícules HDL esdevenen pro-inflamatòries (117,118).

Davant de situacions pro-inflamatòries com és una dieta proaterogènica o una infecció aguda els nivells de ApoA-1, PON1 , PAF-AH i LCAT a les HDL es veuen desplaçats per l'increment de proteïnes prooxidants com el Serum Amiloide-A (SAA) i la ceruloplasmina (119-124).

Per tant, les HDL són partícules heterogènies que formen diferents subfraccions que poden ser identificades en funció de la seva densitat, tamany, càrrega i gràcies a noves tècniques de proteòmica es pot conèixer la seva composició proteica (125-130). D'aquests estudis s'esdevé la conclusió de la importància de l'estudi de la composició de les HDL per investigar la seva

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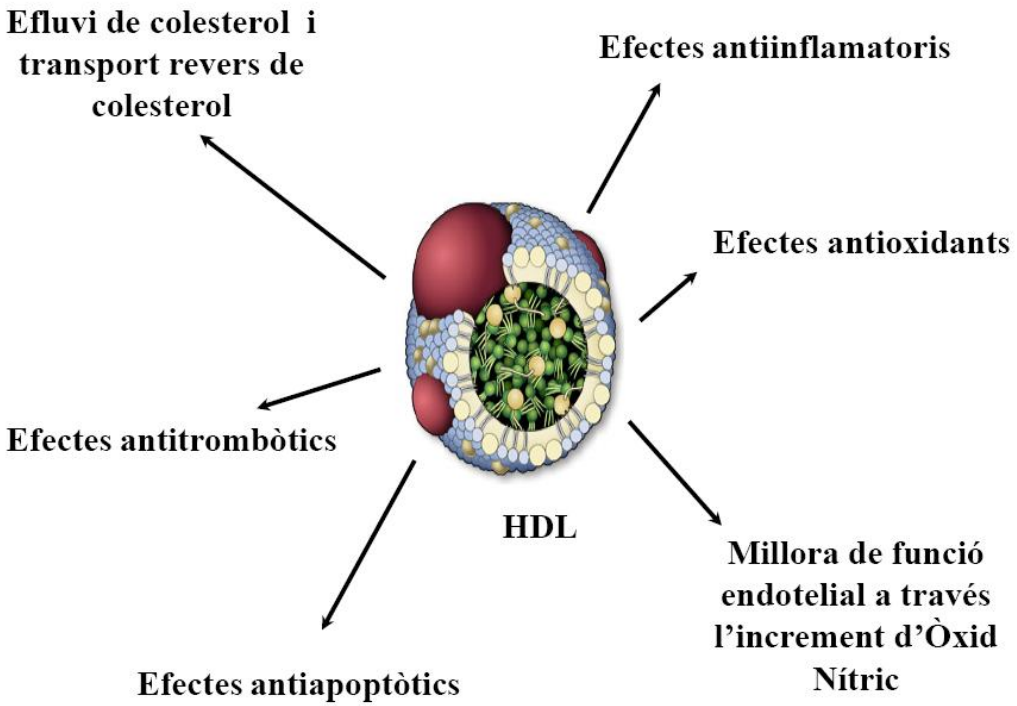
funcionalitat, ja que es modifica en funció de diverses situacions d'estrès inflamatori (132).

En la **figura 7** es resumeixen les diferents propietats antiaterogèniques de les HDL conferida per diferents components i que es poden veure influenciades en diferents situacions clíniques.

Figura 7. Propietats antiaterogèniques de les partícules HDL.



En quant a l'enzim PON1 i la composició de les HDL, en funció del tipus de partícula o subfracció de colesterol HDL contindran diferents concentracions PON1. Hi ha estudis que demostren que les partícules més denses d'HDL_3 contenen més activitat PON1 tot i que els estudis que prediuen l'habilitat de les subclasses d'HDL per prevenir el risc de malaltia coronaria no són concloents (133,134).

2.4. PON1 i malaltia cardiovascular

A partir dels resultats d'aquestes publicacions conferint a PON1 funcions antiaterogèniques, s'han publicat múltiples estudis en humans per investigar l'associació dels genotips de *PON1* i la presència i gravetat de malalties cardiovasculars (135-141). Els resultats d'aquest metanàlisi, finalment no han sigut del tot concloents (142).

Tot i això, s'ha de tenir en consideració que davant d'una malaltia tant complexa com l'arteriosclerosi, les associacions gèniques solen ser febles, requerint mostres de població molt gran. Sí que sembla més clara, l'associació de nivells baixos de l'activitat paraoxonasa i l'aparició d'esdeveniments cardiovasculars. Navab et al. van publicar que pacients amb nivells baixos de HDL però amb activitat paraoxonasa més alta eren menys susceptibles de malalties cardiovasculars que aquells amb HDL més altes i activitats més baixes (143). Un altre estudi poblacional prospectiu va afirmar que els nivells baixos d'activitat paraoxonasa predeien l'aparició d'esdeveniments cardiovasculars (144). Per tant, abans de dissenyar estudis sobre PON1 s'ha de considerar que pot ser tant important estudiar l'*estatus* de PON1 en quant a la seva activitat i concentració en sèrum com els seus polimorfismes genètics (145).

3. HDL i PON1. Funcions dins la immunitat

L'aplicació de noves tècniques dins la branca de la proteòmica com l'espectrometria de masses (125) ha propiciat el descobriment d'altres proteïnes associades a les HDL fins ara no conegudes que li confereixen funcions més amplies en quan a la seva capacitat antiinflamatòria, antioxidant i fins i tot dins la immunitat innata i la regulació del complement, tal i com es reflexa en la següent figura (**Figura 8**) .

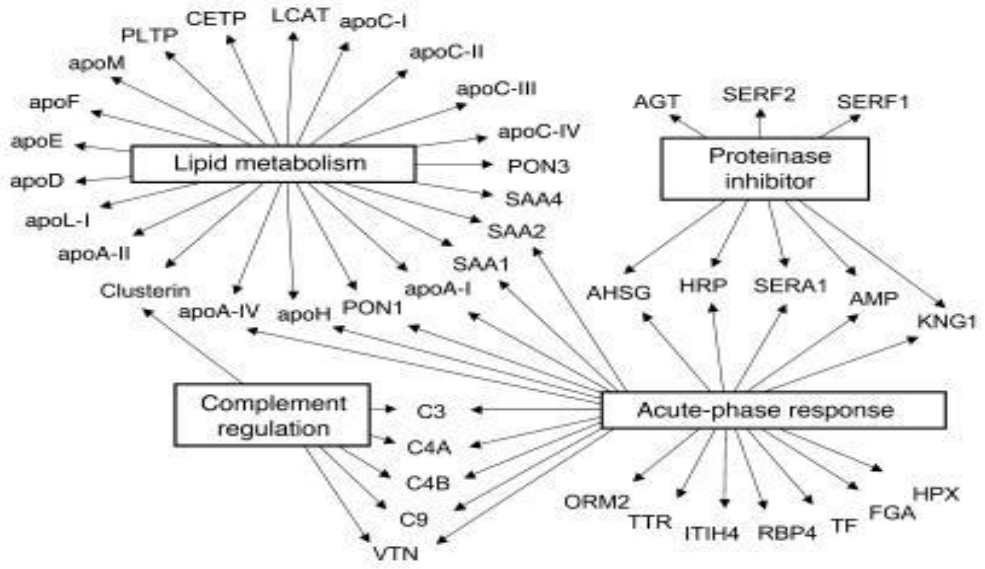


Figura 8. Adaptat de Vaisar i cols The Journal of Clinical Investigation (125)

Un altre exemple d'aquesta capacitat immune de les HDL, ha sigut el descobriment de proteïnes associades a les HDL amb activitat lítica contra el paràsit *Tripanosoma bruceii*. Aquestes lipoproteïnes s'anomenen apoL-I i l'haptoglobin-related protein (HPR) i es troben present en les HDL de primats que estan protegits contra la infecció per aquests paràsit. Aquest treball exposa la idea que les HDL evolutivament formaven part d'un primitiu sistema de defensa innata (146,147).

Altres interessants estudis publicats ja fa més d'una dècada, van demostrar que el plasma té una capacitat innata per protegir contra la virèmia degut a les proteïnes com l'Interferó (IFN), el factor de necrosi tumoral (FNT) i les proteïnes del complement (148).

Existeix un treball que relaciona les paraoxonases amb una funció protectora contra agents infecciosos. El grup de Draganov (149) va demostrar que PON1 degrada una molècula N-3-oxododecanoil homoserina lactona (3OC12-HSL) que té una funció central en la inducció de la regulació de la expressió gènica de *Pseudomona aeruginosa*, mecanisme utilitzat per aquets bacteri per afavorir la seva replicació e infectivitat. Aquesta troballa va ser confirmada *in vivo* pel grup de Zabner i cols. van demostrar que en mosques transgèniques per *PON1*

tipus *Drosophila melanogaster* tenien menys mortalitat per *P aeruginosa* (150,151).

Altres estudis també han demostrat que les partícules d'HDL tenen activitat contra determinats virus com el VHS-1, el virus que provoca la malaltia de Newcastle, el virus que provoca la estomatitis vesicular i el VIH (152,153).

En quant a la infecció pel VIH i la capacitat immune de les HDL, existeixen diferents estudis que han demostrat el factor protector de les HDL en aquesta infecció (154).

Un treball realitzat per Segrest i cols, va demostrar que l'apoA1 i un pèptid sintètic anàleg d'aquesta apoproteïna composta per 18 aa, tenen capacitat per inhibir la formació de sincitis en cultius de cèl·lules CD4 + HeLa infectades pel VIH-1 i que expressaven a la seva superfície proteïnes de l'envoltura del VIH-1, gp120 i gp41. Aquesta inhibició de la formació sincitial protegeix a les cèl·lules no infectades pel virus de ser lisades i destruïdes i per tant disminueix la seva infectivitat (155).

En un altre treball, publicat pel nostre grup, va demostrar que els nivells plasmàtics d'HDL es correlacionaven amb una major probabilitat per mantenir supressió virològica, descrivint també una homologia entre la seqüència d'aminoàcids de la lipoproteïna ApoA1 i una proteïna de la matriu viral del VIH-1 anomenada gp17 (156).

També s'han trobat en diferents estudis correlacions positives directes entre els nivells de CD4 basals i la seva recuperació, amb els nivells de HDL en pacients infectats pel VIH (154,157,158).

4. Infecció pel VIH-1. Complicacions metabòliques

4.1. Relació entre la infecció pel VIH i l'estrès oxidatiu

Els pacients infectats pel VIH presenten un increment de l'estrès oxidatiu que pot ser important per la progressió de la malaltia (159-160). La replicació viral afavoreix l'estimulació de cèl·lules del sistema immune que augmenten la producció de ROS (Reactive Oxygen Species) (161). Els ROS participen en la

inflamació crònica, la replicació viral i l'apoptosi de cèl·lules del sistema immune. La presència de ROS activa l'expressió del factor nuclear-kappa-β (NF-kappa-β) que estimula la replicació viral (162). Aquest augment de l'estat oxidatiu dels pacients també afavoreix la progressió del deterior immunològic mitjançant la deplecció dels limfòcits CD4 induïda per l'apoptosi de les cèl·lules infectades i d'altres que encara no ho estan (163,164).

L'efecte terapèutic de diferents antioxidants mitjançant la suplementació amb diferents vitamines i oligoelements, com el Seleni i la vitamina E, es va proposar com una important estratègia terapèutica sobretot abans de disposar de tractaments antiretrovirals d'alta eficàcia, com actualment es disposen (165,166).

D'altra banda, un estudi dirigit pel grup de Rosenblat M i cols. va demostrar que en ratolins PON1-knockout, el contingut intracel·lular de glutatió reduït estava disminuït i la producció de peròxids lipídics estava incrementada (167).

És a dir, PON1 podria ser important per tal de regular l'estat redox i d'evitar les alteracions associades a l'increment de l'estrès oxidatiu produïda per la infecció pel VIH-1.

4.2. La infecció pel VIH-1 i alteracions del metabolisme lipídic

La prevalença de dislipèmia en pacients infectats pel VIH és del 28-80% (168, 169). Els canvis en el perfil lipídic en la infecció pel VIH es podrien classificar en dos categories. La primera es trobaria en les primeres fases de la infecció, que consta bàsicament de hipetrigliciridèmia, nivells baixos de HDL i LDL amb partícules predominantment més petites i denses (170).

El segon tipus de dislipèmia la trobaríem en aquells pacients que ja han iniciat tractament antiretroviral i es caracteritza per un increment dels nivells de LDL, colesterol total i de triglicèrids. A partir de l'anàlisi de les dades obtingudes de la població *Swiss HIV cohort study*, es va demostrar que la dislipèmia i la hipertrigliciridèmia era 1.7-2.3 vegades més prevalent entre pacients amb

tractament antiretroviral que contenien Inhibidors de la Proteasa (IP) que el grup de pacients sense IP (171,172).

Per tant l'efecte global sobre el perfil lipídic en pacients infectats pel VIH és d'una dislipèmia aterògena amb una reducció significativa de les HDL, un increment dels triglicèrids, de la LDL oxidada i les LDL petites i denses (173-175).

La fisiopatogènesi d'aquestes alteracions lipídiques associades a la infecció pel VIH no són del tot ben conegudes. S'han proposat diferents mecanismes que inclouen un increment de la lipogènesi hepàtica, una alteració de l'aclariment dels lípids del plasma, i altres efectes potencials deguts a l'estat immunològic i inflamatori (176,177).

S'ha demostrat que la infecció pel VIH pot alterar directament el metabolisme de les HDL incrementant la transferència del colesterol de les HDL a les lipoproteïnes que contenen apoB, que són les més aterògenes (178), tal i com s'il·lustra a la **Figura 9.**

La distribució de les subpoblacions de les HDL també es veu influenciat per la infecció i la inflamació crònica com hem esmenat prèviament.

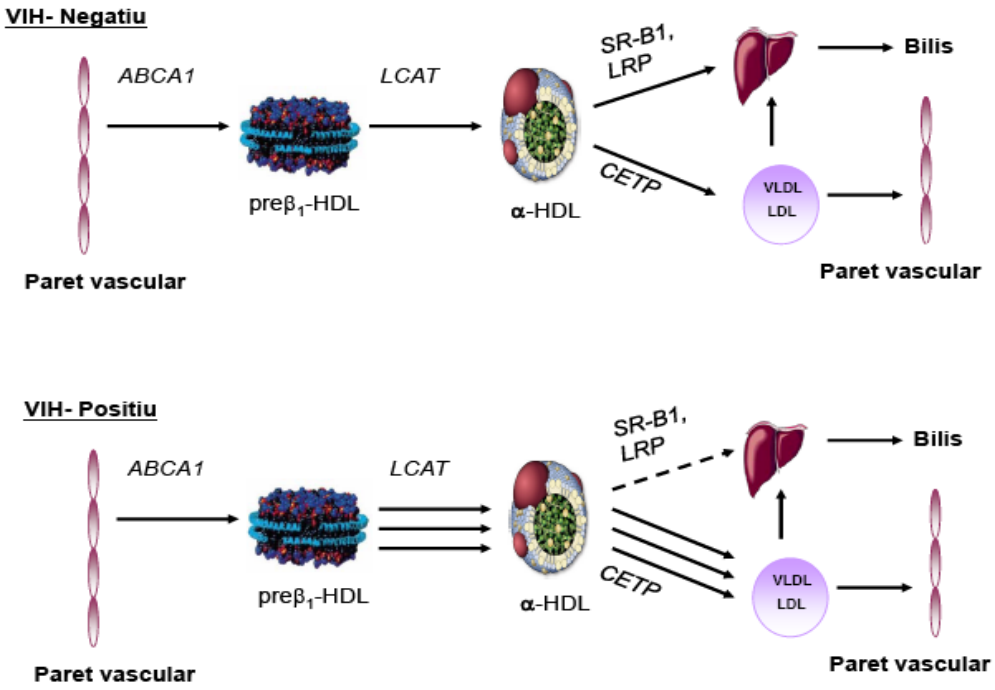


Figura 9. Representació esquemàtica de les alteracions en el transport revers de colesterol durant la infecció pel VIH (178).

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4.3 Malalties cardiovasculars

L'associació entre la presència de malalties cardiovasculars en els pacients infectats pel VIH ha sigut revisada en diferents treballs, tant prospectius com retrospectius. La mortalitat relacionada directament amb la infecció pel VIH ha disminuït des de la introducció del tractament antiretroviral d'alta eficàcia, objectivant un increment de morts relacionades amb malalties cardiovasculars i essent la principal causa de mort en pacients majors de 55 anys (1, 2, 179).

En un estudi retrospectiu realitzat per Triant i cols. (180) en el que es van incloure 3851 pacients infectats pel VIH i més d'un milió de pacients no-HIV del 1996 al 2004, es va observar que la proporció d'infart de miocardi (IAM) era major en pacients VIH respecte la població no infectada ajustada per grups d'edat (**Figura 10**).

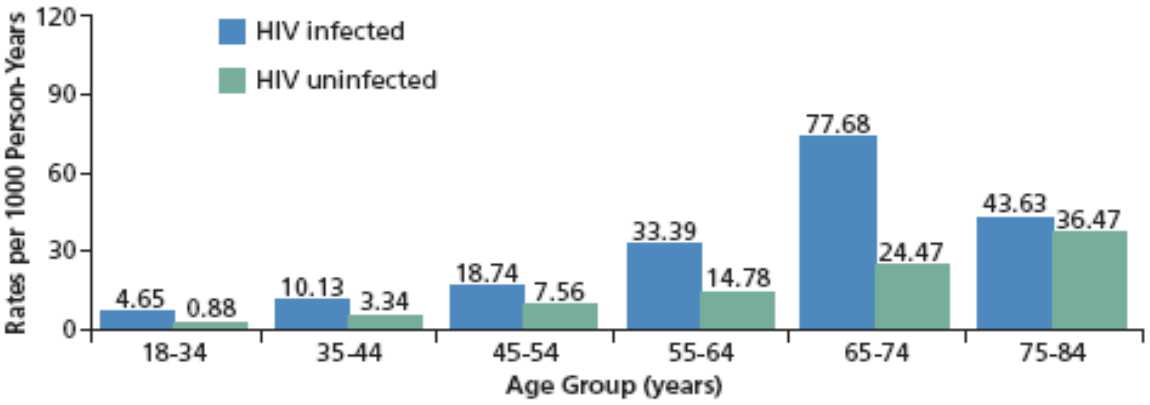

Figura 10. Incidència de IAM en pacients VIH (n = 3851) versus No-VIH (n = 1 044 589) recollits en la base de dades administrativa de Massachusetts des de 1996-2004 (180).

El primer estudi prospectiu sobre la presència de malaltia cardiovascular és el Data Collection on Adverse Events of Anti-HIV Drugs (D:A:D), (181,182) en el que van participar 23468 pacients infectats pel VIH, 126 d'ells (0.5 per cent) van tenir un primer episodi d'IAM durant el seguiment, (incidència de 3.5 per 1000 persona-any). D'acord amb l'estudi D:A:D, els factors de risc cardiovasculars clàssics, com sexe masculí, dislipèmia, edat i tabaquisme

estaven associats a un risc incrementat per desenvolupar un IAM en aquesta població. Això també ha estat ratificat amb altres estudis (183-187) que fins i tot senyalen una major prevalença de factors de risc cardiovascular als pacients infectats pel VIH, com el tabaquisme.

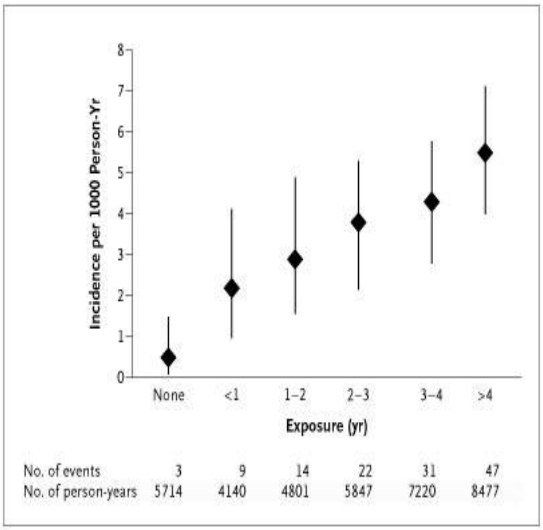

Figura 11. Incidència d'infart de miocardi en pacients infectats pel VIH ajustat pel temps d'exposició al tractament antiretroviral (181).

Una altra de les conclusions més importants de l'estudi D:A:D era que la incidència de IAM augmentava directament en relació amb el temps d'exposició al tractament antiretroviral. (Risc Relatiu, 1.26 [Interval de Confiança del 95%, 1.12 a 1.41] per any addicional d'exposició; *P*<0.001) (**Figura 11**).

Els investigadors d'aquest estudi posteriorment van actualitzar les dades d'aquests resultats, recollint les dades de 23437 pacients, registrant un número absolut de 345 episodis de IAM i una incidència de 3.65/1000 persona-any. Una altre resultat important va ser el fet de trobar que aquest risc relatiu era significativament més gran en pacients que havien estat tractats amb un esquema antirretroviral basat en Inhibidors de la Proteasa (IP), [1.16(1.10-1.23)] comparats amb un règim basat amb Inhibidors de la Transcriptasa Inversa No Anàlegs (ITNAN) [1.05(0.98-1.13)].

L'increment de risc de presentar malalties cardiovasculars en pacients HIV s'ha relacionat també a banda dels factors de risc cardiovasculars clàssics, a la major incidència de dislipèmia secundaria a tractament antiretroviral i a factors associats a la infecció per ella mateixa (188-192).

L'estudi SMART (193) investigava el possible efecte beneficiós de retirar el tractament antiretroviral de forma intermitent en aquells pacients amb CD4+ majors a 350 cèl·lules/μL per tal d'evitar toxicitat farmacològica. Es va veure que aquells pacients que feien "descans" del tractament tenien un increment de malaltia cardiovascular. S'han postulat diferents mecanismes a partir dels quals l'activació de la replicació viral pot interferir en mecanismes proaterogènics, com per exemple la estimulació de la secreció de MCP-1 per cèl·lules musculars llises infectades pel VIH-1 (194) o la inhibició del receptor ABCA-1 per la proteïna viral Nef (195).

La inflamació promoguda per la infecció crònica és un altre mecanisme que pot afavorir la presència d'aterosclerosis accelerada en aquesta població, S'ha objectivat un augment de les citoquines proinflamatòries en pacients infectats pel VIH com la Interleuquina-6, dímer D, Factor de Necrosi Tumoral-alfa (FNT-α), proteïna C-reactiva i molècules d'adhesió com VCMA-1 i ICAM-1(193), totes elles implicades en la fisiopatologia de l'aterogènesi.

Un estudi anatomopatològic va demostrar també que les lesions d'arteries coronaries de necròpsies de pacients infectats pel VIH comparat amb pacients no infectats pel VIH presentaven gran infiltració inflamatòria recordant a les lesions que es presenten en pacients trasplantats (196).

En la **taula 1** es resumeixen els principals estudis que relacionen la presentació d'esdeveniments cardiovasculars en pacients infectats pel VIH.

Taula 1. Estudis observacionals de malalties cardiovasculars amb pacients infectats pel VIH.

Autors	Població d'estudi	Pacients (n)	Duració	VIH vs controls	Efecte TAR
Bozette et al[185]	**Veterans Affairs Hospital system**	36766	1993–2001	No diferències	No diferències entre IP vs no IP
Mary-Krause et al[w186]	**French Hospital Database**	34976	1992–1999	↑ Mortalitat per IAM	Relació amb IP
Currier et al[183]	**California Medicaid**	28513	1994–2000	↑ Risc de cardiovascular en ♂<35 anys ♀ <45 anys	↑ Risc Relatiu IAM si TAR
Friis-Moller et al[181]	**D:A:D**	23468	1999–2005	No controls	↑ Risc Relatiu amb IP
Coplan et al[196]	**Meta-anàlisis estudis fase II/III amb IP**	10986	30 estudis abans del 1999	No controls	No diferències entre IP i ITINAN
Iloeje et al[197]	**HIV Insight (inclosa cohort HOPS)**	7542	1991–2002	No controls	↑ Risc Relatiu amb IP
Holmberg et al[198]	**HOPS**	5672	1993–2002	No controls	No diferències per IP
Rickerts et al[199]	**Frankfurt HIV Cohort**	4993	1983–1998	No controls	Incidència en relació amb TAR
Klein et al [200]	**Kaiser Permanent Northern California**	4159	1996–2001	↑ Ingressos per MCV	No diferències per IP
Obel et al[201]	**Danish National Hospital Registry**	3953	1995–2004	↑ incidència MCV	No comparació IP
Triant et al[180]	**Healthcare based cohort**	3851	1996–2004	Increment MCV	No comparació IP
Kaplan et al	**MACS and Women's Interagency HIV Study**	2386	1984–2003	No controls	No diferències amb IP
Barbaro et al[187]	**Italian cohort**	**1551**	**1999–2002**	**No controls**	**↑IAM amb IP vs no IP**

MCV, malalties cardivoasculars; TAR, Tractament antiretroviral; IAM, infart agut de miocardi; ITINAN, Inhibidors Transcriptasa Inversa No Anàlegs als Nucleòsdis; IP, inhibidors de la proteasa.

4.4. Relació entre limfòcits CD4 i aterosclerosi

Com hem comentat prèviament l'aterosclerosi és una malaltia inflamatòria (203) i la infecció pel VIH està associada amb una activació del sistema immune amb elevació de marcadors inflamatoris (180,204,205). Aquesta activació immunitària no està correlacionada amb un increment dels nivells dels limfòcits CD4+. Nivells baixos de CD4+ al moment del diagnòstic de la infecció pel VIH han sigut relacionats com a predictors de la presència d'esdeveniments cardiovasculars i la progressió d'aterosclerosi (181, 206) Aquestes dades indiquen que tant la immunodepressió com la reconstitució immune posterior poden ser proaterogèniques.

Així doncs, els limfòcits T CD4+ constitueixen factors etiopatogènics en l'aterogènesi (204,205). Entre els components cel·lulars de la placa d'ateroma, les cèl·lules endotelials, els monòcits i les cèl·lules musculars llises són els agents principals. Els limfòcits T CD4+ també promouen la formació d'aterosclerosis amb l'alliberació de citoquines proinflamatòries, com el factor de necrosi tumoral i les interleuquines (207).

Ha sigut publicat prèviament que en la població general la infecció per Chlamydia i Citomegalovirus (CMV) (208) té un efecte estimulador en la formació d'aterosclerosi. En els pacients infectats pel VIH també s'ha associat un increment de la presència d'aterosclerosis carotídea amb un increment de la resposta específica dels limfòcits T contra el virus CMV (209).

Barbaro i cols. (210) van descriure la presència d'una infiltració extensa dels limfòcits T en pacients VIH que recorden a les lesions obtingudes a partir de necròpsies de pacients trasplantats que van patir un IAM, (**figura 12**) resultats també observats per Tabib i cols. on observaven una reacció inflamatòria intensa en plaques ateromatoses de pacients infectats pel VIH. Aquests mecanismes podrien explicar la presència d'arteritis de gran i mitjà tamany observat en alguns pacients infectats pel VIH (196).

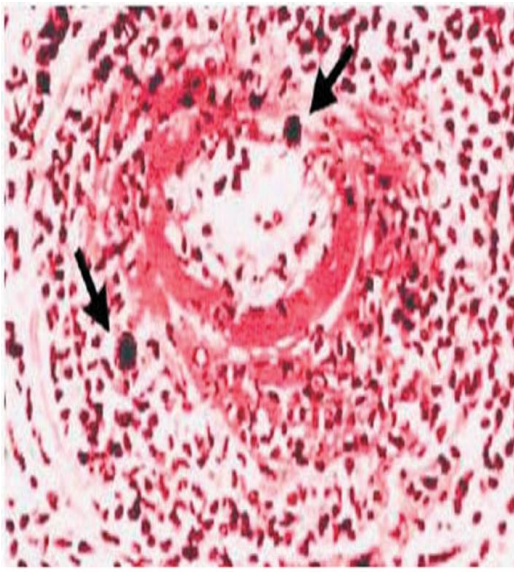

Figura 12. Hibridització in situ de RN-VIH-1. Tall d'una secció transversal de la branca esquerre de l'arteria descendent anterior, d'un pacient VIH amb un Infart de Miocardi. La tinció intensa indica la presencia de seqüències de VIH-1 a l'interior de la íntima i la media (fletxes). Hi ha una infiltració intensa de limfòcits a l'interior de la media i necrosis de la íntima (196).

Aquestes dades, juntament amb les dades obtingudes a partir de l'estudi SMART que observaven també un increment d'esdeveniments cardiovasculars en aquells pacients amb pitjor control immunològic suggereixen que la supressió virològica completa precoç abans d'objectivar deterior immunològic o descens de nivells de CD4 supera els riscos a patir malalties cardiovasculars,degut a un estat proinflamatori mantingut (193).

El fet de disposar de nous fàrmacs antiretrovirals amb perfil lipídic més favorable, millor tolerància i menys efectes secundaris propicia també aqueta possibilitat d'iniciar tractament de forma més precoç. Estudis prospectius sobre la progressió de malaltia cardiovascular i aterosclerosi subclínica serien necessaris per objectivar aquet benefici.

4.5. Síndrome metabòlica i lipodistròfia

La prevalença de síndrome metabòlica és significativament més elevada en pacients VIH que en la població general ajustada per edat i sexe (175) s'han hipotetitzat diferents causes (211), com la presència de dislipèmia, resistència insulínica i lipotoxicitat degut a disfunció mitocondrial (176) i alliberament d'àcids grassos lliures (212,213).

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La lipotoxicitat està associada als fàrmacs antiretrovirals, especialment als Inhibidors de la transcriptasa inversa anàlegs a nucleòtids (ITAN) i als inhibidors de la proteasa (IP) però també a la pròpia infecció pel virus (214,215).

Un nombre important de pacients infectats pel VIH estan afectats per una síndrome d'alteració de la distribució del greix corporal, lipodistròfia, amb greus conseqüències psicològiques i emocionals, degut a la estigmatització dels pacients.

La lipodistròfia es presenta en forma d'acúmuls de greix de localització característica (lipohipertròfia) a nivell de part posterior del coll i abdomen i de desaparició de greix a nivell facial, d'extremitats i glutis (lipoatròfia) tal i com es demostra en la **figura 13.**

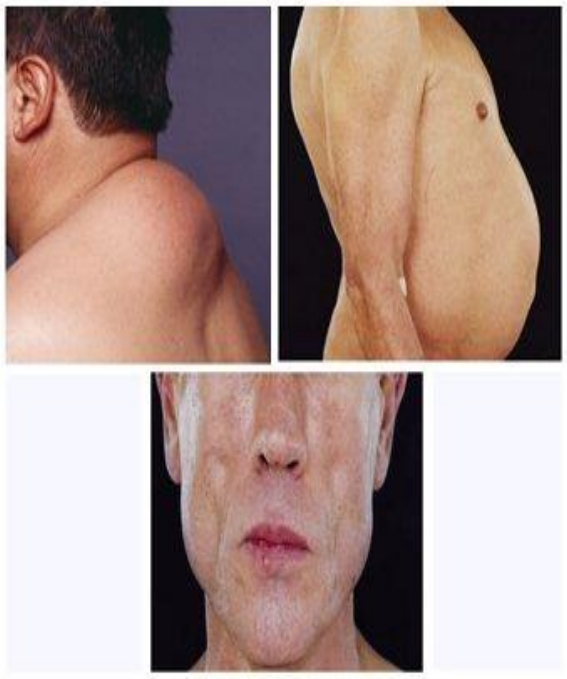

Figura 13. Lipoacúmul a nivell de la zona posterior del coll i greix visceral a la zona abdominal i atrofia de greix subcutani a la zona malar.

De fet la lipodistròfia, presenta grans similituds amb la síndrome metabòlica "clàssica" donat que s'associa amb obesitat visceral abdominal, hipertrigliciridèmia, baixos nivells d'HDL, hipertensió arterial i resistència insulínica. Sembla ser que aquets pacients amb lipodistròfia presenten un major estat proinflamatori i alteracions immunològiques que afavoreixen també l'aparició d'aterosclerosis precoç (216-218).

5. Marcadors d'aterosclerosi subclínica en pacients infectats pel VIH-1. Gruix íntima-mitja (GIM)

Es conegut que la majoria d'esdeveniments cardiovasculars es presenten, no en població general amb una estimació de risc cardiovascular elevat, sinó en aquells amb un risc estimat de grau mitjà (219-221). Aquest fet, juntament amb la necessitat de disposar d'una eina diagnòstica per tal de poder valorar la eficàcia de intervencions terapèutiques en assajos clínics i en la pràctica clínica, ha fet que la possibilitat de disposar d'una eina diagnòstica no invasiva per la determinació de la presència d'aterosclerosi subclínica sigui de gran importància clínica.

La mesura del gruix de la íntima mitja a caròtides (GIM), es va idear a l'any 1986 (222). És un mètode d'estudi d'aterosclerosis subclínica (223) que s'ha correlacionat amb els factors de risc cardiovascular (FRC) i la incidència d'esdeveniments cardiovasculars (ECV), també s'ha utilitzat com objectiu en estudis d'intervenció (224). Un avantatge addicional de la determinació del GIM és la possibilitat de detectar directament la presència de lesió arterioscleròtica en una fase precoç i no només un factor de risc de malaltia cardiovascular (225).

La determinació del gruix de la íntima mitja s'obté realitzant una ecografia-doppler amb una sonda vascular. Es localitza la línea que apareix a la interfase de la llum vascular i la íntima i la interfase entre la capa mitja i la adventícia, tal i com es mostra a la **figura 14**. La distància entre les dues línies constitueix el gruix íntima-mitja (GIM). Per tal de facilitar l'estandardització de la tècnica s'utilitza la localització anatòmica del bulb carotidi abans de la bifurcació de la caròtida comú. Es realitzen determinacions del GIM a l'arteria caròtida interna, bulb carotidi i caròtida comú, realitzant la mitjana dels tres territoris. La digitalització i l'anàlisi de les imatges a partir d'un software (Thicksoft) també ha facilitat la reproductibilitat de la tècnica. L'estudi del GIM a femorals també ha obtingut resultats similars als dels estudis a caròtides (226).

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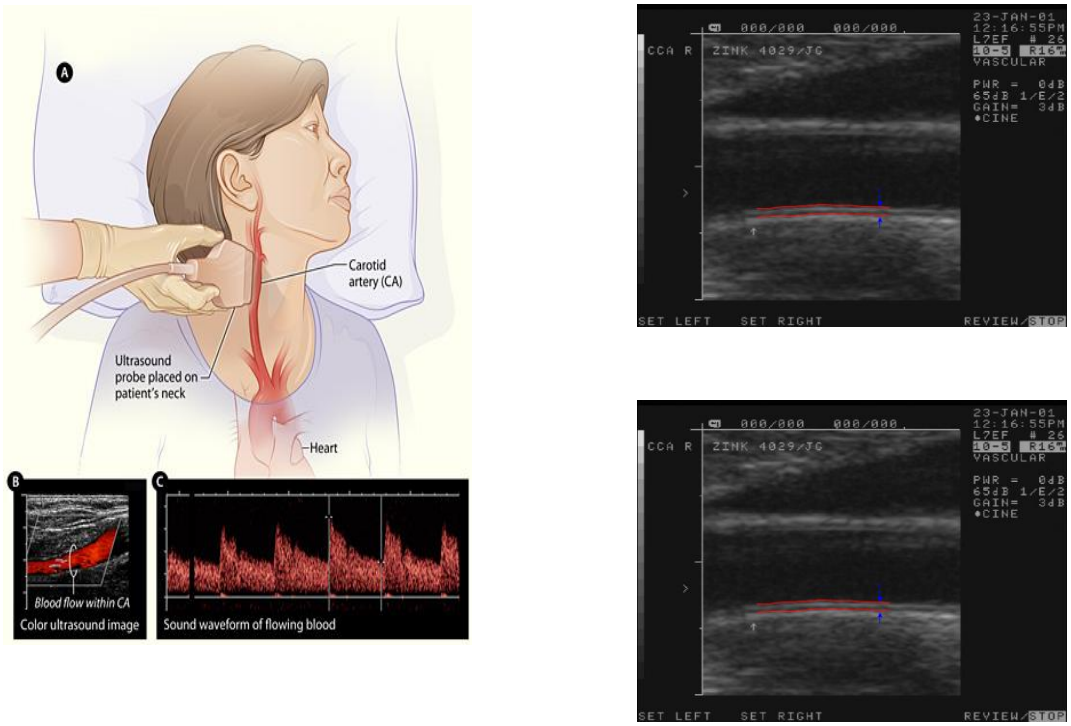

Figura 14. Obtenció de la mesura del GIM a caròtides. (A). Determinació del GIM normal (B) i incrementat (C)

Diferents estudis de GIM de caròtides i femorals s'han associat a aterosclerosi coronària i amb el major número de factors de risc cardiovascular (227)

La capacitat de predir esdeveniments cardiovasculars a partir de la determinació del GIM ha sigut validat a partir de grans estudis observacionals i prospectius (228) O'Leary i cols. van demostrar en una cohort de 5858 participants que a major GIM major probabilitat de presentar un edeveniment cardiovascular, tal i com es mostra a la **figura 15.**

Diferents estudis comparant el GIM en població infectada amb VIH i població no infectada han demostrat que els pacients VIH positius presenten un increment del GIM i una progressió més ràpida del GIM. En la **taula 2** presentem un resum dels principals estudis sobre GIM en pacients VIH.

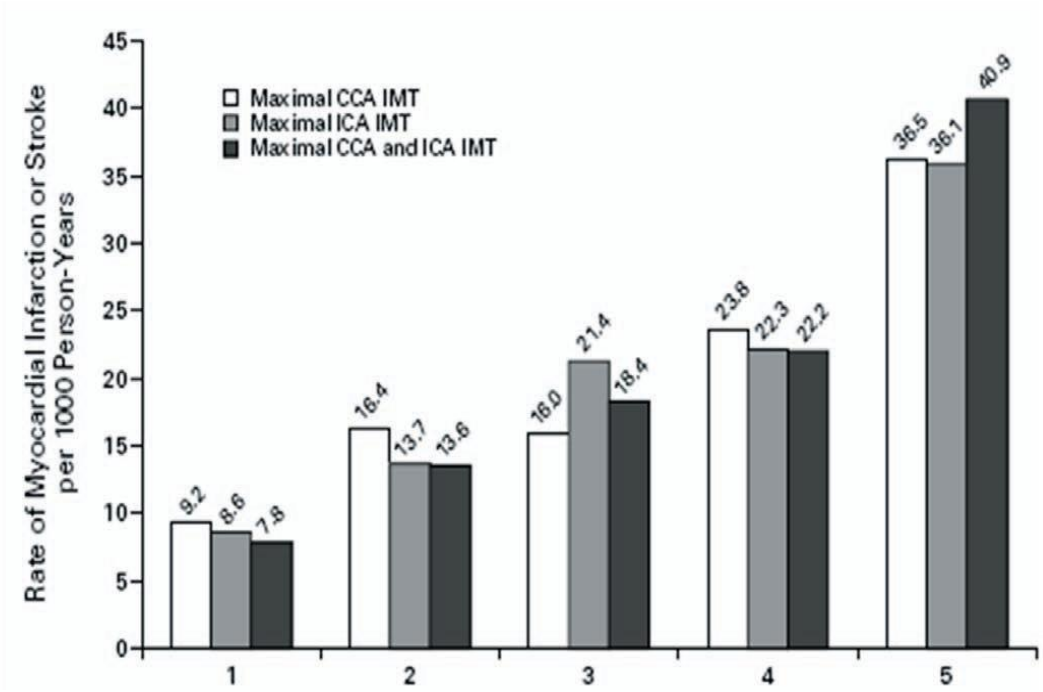

Figura 15. Incidència de IAM d'acord amb quintils de GIM. Els pacients amb el cinquè quintil de GIM tenien un risc relatiu de 3.15 presentar un IAM/ictus de 3.15[(95% IC: 2.19-452)] (228).

Taula 2: Principals estudis de GIM en pacients infectats pel VIH

Autors	Població d'estudi	Pacients	Seguiment	Resultats
Kaplan i cols.	**WIHSMACS**	1931 Casos 859 Controls	No	No diferències
Lorenzo i cols.	**Franckfurt HIV Cohort**	292 Casos 1168 Controls	No	VIH>Controls TAR associat a GIM
Curier i cols.	**ACTG Study A5078**	89 Casos 45 Controls	3 anys	No diferències
De Saint Martin i cols.	**SHIVA**	154 Casos	No	GIM depèn de l'edat, TAS, TG, i tractament amb IP
Hsue i cols.	**SCOPE**	93 Casos 36 Controls	No	VIH> Controls
Maggi i cols.	**PREVALEAT**	133 Casos	2 anys	Pacients amb IP tenen més IMT
Mangili i cols.	**Nutrition for Healthy Living Study**	327 Casos	No	No diferències per TAR
Jerico i cols.	**Barcelona**	132 Casos	No	IMT>0.8mm o placa en 41.7%
Currier i cols.	**ACTG Study A5078**	89 Casos 45 Controls	No	No diferències
Hsue i cols.	**SCOPE**	143 Casos 63 Casos	1 any	Progressió IMT >VIH
Depairon i cols.	**Swiss HIV Cohort Study**	168 Casos 68 Controls	No	VIH>Controls
Maggi i cols.		102 Casos 104 Controls	No	VIH>Controls

ACTG, AIDS Clinical Trials Group; TAR: Tractament antiretroviral, MACS, Multicenter AIDS Cohort Study;; PREVALEAT, Premature Vascular Lesions and Antiretroviral Therapy Study; SCOPE, Study of the Consequences of the Protease Era; SHIVA, Study of HIV and Atherosclerosis; WIHS, Women's Interagency HIV Study. IP: Inhibidors de la Proteasa; TAS: tensió arterial sistòlica;

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HIPÒTESI i OBJECTIUS

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HIPÒTESI

La relació entre la infecció pel VIH i l'estrès oxidatiu és tal que PON1 podria tenir una funció amb repercussions clíniques i convertir-se en un possible objectiu terapèutic.

Els genotips de *PON1* podrien contribuir a la possible funció immune de les HDL amb repercussions en l'evolució immunològica i virològica dels pacients infectats pel VIH.

La relació entre la presència d'aterosclerosi precoç i lipodistròfia en pacients infectats pel VIH està associada a l'elevada prevalença de dislipèmia però també està en relació a un increment de l'estrès oxidatiu i a un estat inflamatori. Els genotips de *PON1* amb diferent capacitat antioxidant i antiinflamatòria podrien tenir influència en l'aparició d'aquestes complicacions metabòliques.

L'activitat i la concentració de PON1 podria ser utilitzada com a marcador serològic predictiu per la presència d'aterosclerosi subclínica en els pacients infectats pel VIH-1 i ser d'utilitat per l'avaluació del risc cardiovascular dels pacients.

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OBJECTIUS

1. Investigar l'existència de diferències genotípiques de *PON1* en pacients infectats pel VIH-1 respecte una població control.

2. Investigar les diferències entre l'activitat i concentració de PON1 respecte una població control.

3. Investigar la influència dels genotips de *PON1* sobre l' activitat i la concentració sèrica de l'enzim en pacients infectats pel VIH-1.

4. Investigar la influència de la presència de lipodistròfia i coinfecció pel VHC sobre l'activitat i la concentració de PON1.

5. Investigar la relació entre els genotips de *PON1* i la evolució immunològica i virològica dels pacients infectats pel VIH-1.

6. Investigar la influència dels genotips de *PON1* en l'aparició de dislipèmia, síndrome metabòlica, lipodistròfia i malaltia vascular.

7. Investigar la utilitat de l'activitat i concentració sèrica de PON1 per l'avaluació de risc cardiovascular i la presència d'aterosclerosis subclínica en pacients infectats pel VIH-1.

8. Investigar la influència de la infecció pel VIH-1 sobre l'activitat antiinflamatòria i antioxidant de l'enzim PON1 utilitzant diferents substrats.

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MATERIAL I MÈTODES

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1. Participants i Disseny

ESTUDI 1

Es va realitzar un estudi transversal descriptiu per estudiar l'existència de diferències entre els genotips de *PON1* i el seu producte enzimàtic mesurat per l'activitat i la concentració de l'enzim.

Van ser inclosos per l'estudi 212 pacients infectats pel VIH-1 (146 homes i 66 dones) que provenien de la consulta especialitzada de l'Hospital Universitari Sant Joan de Reus. D'aquests pacients 129 estaven co-infectats pel VHC.

Els criteris d'exclusió van ser el tenir menys de 18 anys, una alteració de la funció renal o tenir una malaltia definitòria de SIDA al moment de la seva possible inclusió

La presència de lipodistròfia va ser definida per la presència d'alteracions de la distribució del greix corporal que podien ser clarament reconegudes pels propis pacients i confirmades pel metge. Aquests canvis inclouen lipoatròfia subcutània (pèrdua de greix a nivell malar, venes superficials prominents a nivell extremitats, disminució de volum a nivell de glutis) i obesitat central (creixement de perímetre abdominal, increment de la mida mamària o acumulació de greix a la nuca i al coll).

El grup control va estar format per 409 voluntaris sans participants en un estudi epidemiològic desenvolupat a la nostra àrea geogràfica. Els detalls d'aquesta població van ser publicats prèviament (229). En resum es tracta de participants d'origen caucàsic provinent de la regió de Catalunya (194 homes i 215 dones) amb una mitjana d'edat de 42 anys (rang de 19 a 75 anys). Els voluntaris eren sans aparentment sense evidència d'insuficiència renal, dany hepàtic, neoplàsia o alteracions mentals.

Es va obtindre una mostra de sang en dejú de tot els participants. Les mostres van ser congelades a -80ºC fins la determinació de les variables bioquímiques estudiades.

ESTUDI 2

Es va realitzar un estudi cas-control per investigar les diferències entre les distribucions genotípiques dels gens de *PON1* en la població de pacients infectats pel VIH-1 respecte una població no infectada.

Com a grup control es van utilitzar la cohort de voluntaris sans inclosos en l'estudi 1 que va ser ampliada fins a 633 voluntaris (339 dones i 229 homes) amb una mitjana d'edat de 45 anys (rang de 18 a 81 anys).

La cohort dels pacients que es van incloure a l'estudi (n = 234; 72 dones , 162 homes, amb una mitjana d'edat de 38.7 anys (rang de 20 a 66 anys) provenien de la mateixa consulta especialitzada de SIDA que s'ha comentat a l'estudi 1

Es va realitzar un segon estudi cas-control per tal d'analitzar les associacions entre els haplotips de *PON1* i les variables metabòliques, immunològiques i virològiques estudiades incloent el grup dels pacients infectats pel VIH.

ESTUDI 3

Es va realitzar un estudi transversal observacional. Es van incloure 187 dels pacients infectats pel VIH inclosos als estudis previs que se'ls va realitzar la determinació del gruix intima-mitja a caròtides i femorals i no tenien història personal de malaltia cardiovascular.

Es va calcular el risc cardiovascular estimat als 10 anys utilitzant Framingham Risk Score. Es van obtenir tres categories en funció del risc baix <10%, moderat 10-20% o alt >20%.

2. Requeriments ètics

Els estudis van ser aprovats pel Comitè Ètic de l'Hospital Universitari Sant Joan de Reus.

Les dades van ser codificades per tal d'assegurar la confidencialitat i l'anonimat dels pacients.

A tots els pacients se'l va lliurar un consentiment informat que va ser signat abans de ser inclosos en l'estudi.

Tots els procediments es van realitzar d'acord els principis i la correcta pràctica clínica recollits a la Declaració de Hèlsinki.

3. Determinacions analítiques

I. Paràmetres bioquímics generals

- ✓ **Variables immunològiques i virològiques:**
  a. Càrrega viral de VIH-1: realitzada mitjançant el Cobas® TaqMan HIV-1 assay. (Roche, Basilea, Suïssa)
  b. Poblacions limfocitàries (LT CD4+ i CD8+) mitjançant citometria de flux (Beckman-Coulter, Fullerton, CA, EUA)
  c. Anticossos contra VHC: Immunoassaig mitjançant analitzador automatitzat (Abbot Axsym, Abbot Park IL, EUA)
  d. β2-Microglobulina: marcador de destrucció limfocitària i de progressió de la infecció pel VIH. Determinat per immunoassaig turbidimètric (Beckman-Coulter, Fullerton, CA, EUA).

- ✓ **Variables Metabòliques**
  a. Colesterol –HDL: realitzat per un mètode homogeni (230)
  b. Concentració colesterol total, triglicèrids i glucosa: es van utilitzar mètodes estàndards (Beckman-Coulter, Fullerton, CA,EUA)
  c. Concentració de colesterol LDL: calculat mitjançant la fórmula de Friedewald
  d. Concentració LDL oxidada: ELISA utilitzant un kit comercialitzat (Mercodia, Uppsala, Suïssa)
  e. Concentració de apolipoproteïnes-A-I, apolipoproteïnes-A-II i IL-6: Van ser determinats per immunoassaig turbidimètric (Beckman-Coulter, Fullerton, EUA)

f. Proteïna C-reactiva: Es va utilitzar un mètode d'alta sensibilitat (Beckman- Coulter, Fullerton, EUA)
g. Concentració MCP1: Determinada per ELISA (Human MCP-1 ELISA Development Kit, Prepotech Londres, UK)

II. Determinacions dels nivells circulants de PON1

✓ Activitat esterasa de PON1

Es va determinar mesurant la taxa d'hidròlisi del substrat paraoxó a 410 nm i 37ºC, en un tampó glicina 0.05 mM, pH 10.5, complementat amb 1mM de clorur de calci (CaCl2) (154). L'activitat es va expressar en U/L (1 U equival a 1 µmol de paraoxó hidrolitzat per minut). El substrat paraoxó es va preparar de nou per a cada determinació.

✓ Activitat lactonasa de PON1

L'activitat lactonasa es va analitzar mesurant la hidròlisi del substrat sintètic 5-tiobutil butirolactona (TBBL), substrat i mètode cedits pel Dr. Tawfik, del Weizmann Institute of Science (Rehovot, Israel) (162). Els reactius utilitzats contenien 1mM de CaCl2, 0.25 mM de TBBL i 0.5 mM de l'àcid 5,5'-ditio-bis-2-nitrobenzoic (DTNB), en 0.05 mM del tampó Tris- HCl pH 8. Els canvis en l'absorbància van ser monitorades a 412 nm durant 5 minuts. L'activitat també es va expressar en U/L (1 U equival a 1 mmol de TBBL hidrolitzat per minut).

✓ Concentració de PON1

La concentració sèrica de PON1 va ser determinada per un ELISA no comercial. L'anticòs anti-PON1 va ser cedit pels Drs. Mackness (Universitat de Manchester, Regne Unit). Es va obtenir inoculant conills amb el pèptid derivat de la seqüència específica per PON1 madura (CRNHQSSYQTRLNALREVQ).

4. Variables de genotipatge

Estudi 1

Partint de plasma EDTA, es van aïllar leucòcits, a partir dels quals es va obtenir DNA genòmic (Puregene DNA Isolation reagent set, Gentra Systems Inc., Minneapolis, MN, Estats Units). Posteriorment, es van determinar els polimorfismes *PON1-$_{107}$*, *PON1$_{192}$* *i* *PON1$_{55}$* per amplificació per PCR i isotipatge per restricció enzimática (76).

Estudi 2

Es va obtenir DNA de leucòcits aïllats mitjançant mètodes descrits a l'apartat anterior. Després es van analitzar 7 SNP escollits entre els més importants del gen de *PON1* (*PON1$_{192}$*, *PON1$_{55}$*, *PON1$_{-162}$*, *PON1$_{-832}$*, *PON1$_{-909}$*, *PON1$_{-1076}$* i *PON1$_{-1741}$*) i també el genotip de *MCP-1$_{2518}$*. Els SNPs es van analitzar segons la tecnologia del Iplex Gold MassArrayTM (Sequenom Inc., San Diego, CA, Estats Units) al CEGEN (Centro Nacional de Genotipado, Universitat Pompeu Fabra, Barcelona, Espanya).

5. Mesura del gruix íntima-mitja a caròtides

Es van realitzar ecografies als territoris femorals i carotidis en 183 dels pacients. Aquestes mesures van ser realitzades utilitzant els mateixos protocols pels mateixos investigadors. Es va utilitzar l'ecògraf GE Logiq 700 amb una sonda ecogràfica de 7-10 MHz. Es van identificar tres territoris a l'arteria caròtida on es van realitzar les mesures. L'arteria caròtida comú (1cm a nivell proximal de la bifurcació), el bulb carotidi (a la bifurcació) i l'arteria caròtida interna (1 cm distal a la bifurcació). Es van obtindre i digitalitzar les imatges de cada pacient i es va calcular la mitjana a partir de les determinacions dels tres territoris. La presència d'aterosclerosis subclínica es va definir a partir d'una mitjana de GIM > 0.8 mm o la presència d'una placa. Una placa es va definir a partir d'un engruiximent >1.5 mm o una estructura focal que envaeix la llum vascular al menys 0.5 mm o un 50% del valor de la GIM al territori subjacent.

6. Anàlisis estadístiques

Tots les anàlisis estadístiques van ser realitzats utilitzant el programa SPSS de la versió 12.0 a la 17.0.

La significació estadística mínima per cada test es va es va considerar a partir de $P<0.05$.

a. Es van utilitzar mètodes estàndard (Kolmogorov-Smirnov i Shapiro-Wilks) per comprovar si les variables seguien una distribució normal.
b. Les freqüències al·lèliques van ser estimades pel mètode gene-counting. Es va realització el tes de χ^2 l'equilibri de Hardy –Weindberg per analitzar les diferències en la distribució genotípica entre els grups.
c. Les diferències entre dos grups es van analitzar amb el test t-Student (paramètric) o amb el test U-Mann-Whitney (no paramètric). Les diferències entre múltiples grups es van analitzar amb l'ANOVA.
d. Per avaluar el grau d'associació entre dues variables es van utilitzar els coeficients de correlació de Pearson (paramètric) i Spearman (no paramètric) o el test de Kruskall Wallis (categòric).
e. Es van crear models de regressió logística múltiple per estimar la capacitat de grups de variables per predir les variables estudiades o la presència o absència de malaltia.
f. En el cas dels anàlisis d'expressió d'SNPs, de l'**estudi 2** es va utilitzar el programa Haploview 4.0 per examinar l'equilibri de Hardy-Weinberg. Les estimacions de desequilibri de lligament entre SNPs es van calcular mitjançant el test de Fisher. Per tal de determinar la subseqüent distribució de les freqüències d'haplotips en la població d'estudi, es van utilitzar els programes PHASE (versió 2) i SNPator (http://www.snpator.com).

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RESULTATS

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Serum paraoxonase-1 activity and concentration are influenced by human immunodeficiency virus infection

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Abstract

Background: Higher high-density lipoprotein concentrations are associated with a better disease course in HIV-infected patients. Paraoxonase-1, an enzyme contained within high-density lipoproteins, is thought to hydrolyse oxidised lipids. The aim of the present study was to investigate the relationships between HIV infection and the circulating activity and concentration of paraoxonase-1, and the concentration of high-density lipoproteins, apolipoprotein A-I and oxidised low-density lipoproteins.
Methods: We studied patients with HIV infection ($n = 212$) and healthy subjects ($n = 409$). In all the participants we measured the relevant biochemical and genetic variables. The statistical associations between these variables and paraoxonase-1 activity and concentration were assessed using multiple linear regression analysis.
Results: Serum paraoxonase-1 activity was decreased ($P < 0.001$) and its concentration was increased ($P = 0.017$) in HIV-patients compared to the controls. HIV infected patients had lower HDL-cholesterol and apolipoprotein A-I concentrations. Multivariate regression analysis showed that serum paraoxonase-1 activity was associated with the CD4+ T lymphocyte count ($P < 0.05$), apolipoprotein A-I ($P < 0.001$), and paraoxonase-1 genetic polymorphisms ($P < 0.001$). Paraoxonase-1 concentration was associated with that of serum β-2-microglobulin ($P < 0.001$).
Conclusions: Both, paraoxonase-1 activity and concentration were influenced by HIV-infection and these were related to alterations in HDL composition and the immunological status of the patients.

Keywords: High-density lipoproteins; HIV-infection; Paraoxonase-1

1. Introduction

Patients affected by the human immunodeficiency virus (HIV) infection often develop long-term pro-atherogenic metabolic alterations. This phenomenon may be explained by the infection itself or by the secondary effects of antiretroviral therapies [1,2]. High-density lipoprotein (HDL) concentration is known to be decreased in HIV-infected patients [3]. This lipoprotein is considered part of the innate immune system. Changes in lipoprotein metabolism that occur during the host's response to infection include anti-infective, anti-inflammatory and anti-oxidant effects that are part of the host's defence systems [4,5]. Higher HDL concentrations have been associated with a better disease-course in HIV-patients undergoing antiretroviral treatment [6].

Paraoxonase-1 (PON1) is a hydrolase that circulates tightly bound to HDL in plasma and has been postulated to

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hydrolyse oxidised lipids in low-density lipoproteins (LDL), HDL, and homocysteine-thiolactone [7–11]. The relationship and equilibrium between PON1 and HDL can be altered in the presence of pathological conditions such as diabetes [12] and coronary artery disease [13]. PON1 is mainly synthesized by the liver and its measurement has been proposed as a marker of hepatic function [14].

The *PON1* gene contains a number of functional polymorphisms in the coding, as well as the promoter, regions which determine the levels of enzyme activity and, probably, the ability to protect against lipid peroxidation [9]. Some of the most-studied among these polymorphisms are a *Gln* → *Arg* substitution at position 192 (termed the Q and R alleles, respectively), a *Met* → *Leu* substitution at position 55 (termed M and L alleles, respectively), and the promoter polymorphism *C*–107*T* substitution [see reference [15] for a recent review].

There is a paucity of data on PON1 status and its genotype distributions in patients infected with HIV. Our hypothesis is that the relationship between oxidation and HIV infection is such that PON1 may play a role in this disease; a role that may be susceptible to therapeutic modification and, consequently, to clinical improvement of the patient. Hence, the key objective of the present study was to investigate the influence of the HIV infection on serum PON1 activity and concentration, and their relationships with the circulating concentrations of HDL, apolipoprotein A-I (apo A-I), oxidised LDL (ox-LDL) and the *PON1* genetic polymorphisms.

2. Participants and methods

2.1. Study population

From among the HIV-infected patients attending our clinic, 212 (146 men, 66 women; mean age 38.8 years; range 22–66) accepted the invitation to participate in the present study. Of these patients, 129 were co-infected by the hepatitis C virus (HCV). The exclusion criteria were age <18 years, or renal function impairment defined as creatinine levels higher than 106 μmol/L, or having an AIDS-related opportunistic disease at the time of the study. Forty-five patients had lipodystrophy, defined as the presence of body-fat changes that could be clearly recognized by the patients themselves and confirmed by the physician. These changes included subcutaneous lipoatrophy (hollow cheeks, prominent superficial veins in the limbs, or flattening of the buttocks) and central obesity (increased abdominal girth, breast enlargement, or dorsocervical fat pad) [16]. The main clinical characteristics of the HIV-infected patients are summarised in Table 1. The control group consisted of 409 healthy volunteers who participated in an epidemiological study, the details of which have been previously reported [17]. A fasting venous sample blood was obtained from all the participants and serum was stored at −80 °C until measurements were performed.

Table 1
Clinical characteristics of the HIV-infected patients ($n = 212$)

Characteristic	*n*
Age (years (S.D.))	38.8 (6.9)
Gender (*n* [%])	
Male	141 [67]
Female	71 [33]
Risk factors for HIV (*n* [%])	
Intravenous drug use	124 [59]
Heterosexual contact	63 [29]
Male homosexual contact	25 [12]
Time of sero-prevalence (years (S.D.))	4.29 (3.55)
HCV infection (*n* [%])	129 [61]
Lipodystrophy (*n* [%])	45 [21]
Treatment scheme (*n* [%])	
Non nucleoside analogues	96 [46]
Nucleoside analogues	174 [84]
Protease inhibitors	132 [64]
Patients naïve to treatment scheme (*n* [%])	
Non nucleoside analogues	110 [53]
Nucleoside analogues	27 [13]
Protease inhibitors	74 [36]
CD4+ T lymphocytes (cells/mm^3, mean (S.D.))	447.6 (286.6)

All the participants provided fully informed consent to participation in the study on the understanding that anonymity of all data is guaranteed. The study was approved by the Ethics Committee of the Hospital Universitari de Sant Joan de Reus.

2.2. Virological and immunological measurements

Plasma viral load was measured with the Amplicor HIV-1 monitor assay (Roche, Basel, Switzerland) and CD4+ T-cell and CD8+ T cell counts by FAC scan flow cytometry (Becton-Dickinson, Madrid, Spain). Antibodies against HCV were measured by microparticle-enhanced immunoassay in an automated analyser (Abbott Axsym, Abbott Park IL, USA). β-2-Microglobulin, a marker of lymphocyte destruction and progression of HIV-infection [18] was measured by a turbidimetric immunoassay (Biokit, Barcelona, Spain).

2.3. Metabolic parameters and PON1 status

HDL-cholesterol was determined by a homogeneous method [19]. PON1 activity was analysed by measuring the rate of hydrolysis of paraoxon at 37 °C [14]. PON1 concentration was determined by an in-house enzyme-linked immunosorbent assay (ELISA) [20]. ox-LDL concentration was measured by enzyme-linked immunoassay (Mercodia, Uppsala, Sweden). Apo A-I concentration was analysed by a turbidimetric immunoassay (Beckman-Coulter, Fullerton, CA). Serum cholesterol and triglycerides were analysed by standard methods (ITC Diagnostics, Barcelona, Spain). LDL-cholesterol was calculated by the Friedewald formula [21].

2.4. PON1 genotyping

Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set; Gentra) and the *PON1*$_{192}$, *PON1*$_{55}$, and *PON1*$_{-107}$ polymorphisms of the *PON1* gene were analysed by PCR amplification and restriction isotyping, as we have described previously [17].

2.5. Statistical analysis

Normality of distributions was tested with the Kolmogorov-Smirnov test. Allele frequencies were estimated by the gene-counting method. Hardy–Weinberg equilibrium, differences in genotype distributions and allele frequencies between groups were tested by the χ^2 test. Differences in PON1 activities and concentrations between control subjects and HIV-infected patients were determined by non-parametric tests (Kruskal–Wallis and Mann–Whitney *U* tests) and were adjusted for the polymorphisms studied. Results are expressed as means and the 95% confidence intervals in parenthesis. We used the Spearman's ρ test to analyse the correlation between PON1 activity, PON1 concentration, HDL concentrations, ox- LDL and apo A-I. A multiple linear regression model was fitted to evaluate the factors that were independently associated with PON1 activity and concentrations in HIV-infected patients. The variables included in the model were age, gender, positivity for HCV antibodies, body mass index (BMI), CD4+ T lymphocyte cell count, apo A-I, ox-LDL, HDL-cholesterol, β-2-microglobulin and *PON1* genotypes. All statistical analyses were performed using the SPSS statistical package (version 12.0).

3. Results

3.1. Relationships between PON1 activity, concentration, and lipoprotein profile

Results are summarised in Table 2. HIV-infected patients had significantly lower serum PON1 activity compared with the control group ($P < 0.001$). Conversely, the patients had a significantly higher serum PON1 concentration ($P = 0.017$). Patients had significantly lower cholesterol, LDL and HDL-cholesterol and apo A-I concentrations than the control group ($P < 0.001$). Serum triglyceride concentration was higher in HIV-infected patients than in the control group ($P < 0.001$). There were no significant differences in ox-LDL concentrations in HIV-infected patients with respect to the control group ($P = 0.940$). In the control group, HDL-cholesterol concentration showed significant direct correlations with serum PON1 activity ($\rho = 0.217$; $P < 0.001$) and apo A-I concentration ($\rho = 0.788$; $P < 0.01$) and inverse correlations with PON1 concentration ($\rho = -0.125$; $P < 0.05$) and ox-LDL ($\rho = -0.223$; $P < 0.05$). Serum PON1 activity was also inversely correlated with ox-LDL ($\rho = -0.150$; $P < 0.05$) in these subjects. Conversely, HIV-infected patients had significant associations only between HDL-cholesterol and apo A-I ($\rho = 0.647$; $P < 0.01$) and between HDL-cholesterol and ox-LDL ($\rho = -0.18$; $P < 0.05$).

Table 3
Frequency distribution of *PON1* genotypes

Isoform	Control subjects ($n = 409$)	HIV-infected patients ($n = 212$)
PON1$_{192}$ (*n* [%])		
QQ	198 [48.4]	114 [53.7]
QR	179 [43.8]	78 [36.7]
RR	32 [7.8]	20 [9.4]
PON1$_{55}$		
LL	161 [39.4]	73 [34.4]
LM	191 [46.7]	105 [49.5]
MM	57 [13.9]	34 [16.0]
PON1$_{-107}$		
CC	88 [21.5]	55 [25.9]
CT	201 [49.1]	94 [44.3]
TT	120 [29.3]	63 [29.7]

3.2. PON1 genotype distributions

The allele distributions of the *PON1*$_{192}$, *PON1*$_{55}$ and *PON1*$_{-107}$ polymorphisms followed the Hardy–Weinberg equilibrium (χ^2 $P = 0.25$, 0.91 and 0.15, respectively). We observed linkage disequilibrium between the polymorphisms of *PON1*$_{55}$ and *PON1*$_{192}$, and the *PON1*$_{55}$ with the *PON1*$_{-107}$ polymorphism. There were no significant differences in the *PON1* genotype distribution between the control group and the HIV-infected patients (Table 3).

Table 2
Serum PON1 and lipoprotein profile in the control group ($n = 405$) and HIV-infected patients ($n = 212$)

Parameter	Control group	HIV-infected patients	*P*
PON1 activity (U/L)	410.7 (395.6–425.9)	335.7 (314.5–356.8)	<0.001
PON1 concentration (mg/L)	98.0 (90.2–105.9)	137.5 (116.8–158.1)	0.017
Cholesterol (mmol/L)	5.26 (5.15–5.36)	4.88 (4.70–5.06)	<0.001
HDL-cholesterol (mmol/L)	1.53 (1.49–1.57)	1.18 (1.12–1.24)	<0.001
LDL-cholesterol (mmol/L)	3.13 (3.04–3.22)	2.75 (2.60–2.90)	<0.001
Triglycerides (mmol/L)	1.31 (1.21–1.41)	2.68 (2.08–3.29)	<0.001
Apolipoprotein A-I (g/L)	1.64 (1.61–1.70)	1.39 (1.30–1.42)	<0.001
ox-LDL (U/L)	85.78 (81.80–89.70)	86.02 (81.32–90.71)	0.940

Results are presented as means and 95% CI (in parenthesis).

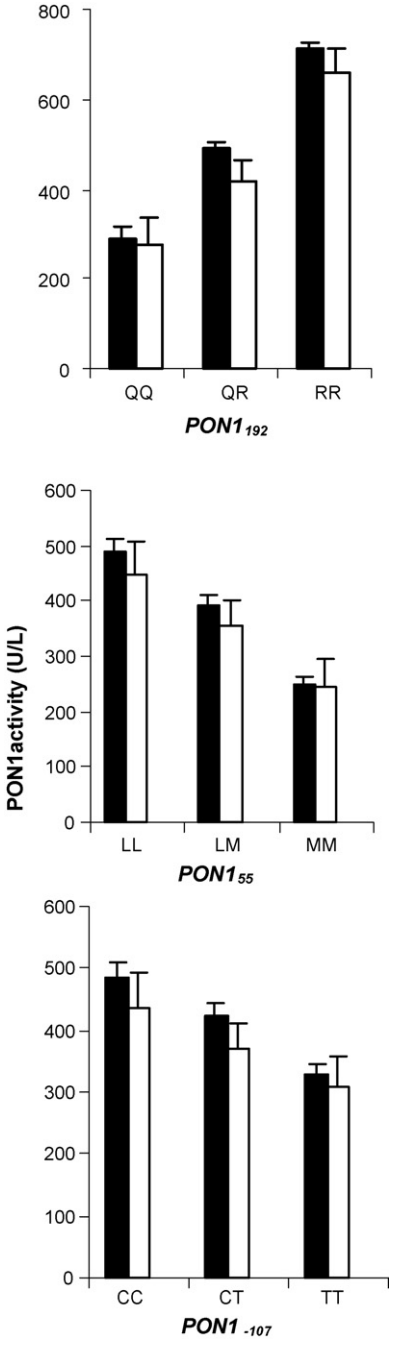

Fig. 1. Serum PON1 activity segregated with respect to the different polymorphisms in the control group (full bars) and HIV-infected patients (empty bars).

When the enzyme activities were segregated with respect to genotypes, we observed that the *PON1* polymorphisms affected the enzyme activity in a similar manner in HIV-infected patients as in the control subjects (Fig. 1). The order of activity was consistent in the three groups QQ < QR < RR for the $PON1_{192}$ polymorphism; MM < ML < LL for the $PON1_{55}$ polymorphism; and TT < TC < CC for the $PON1_{-107}$ polymorphism ($P < 0.001$).

Table 4
Variations of PON1 activity and concentration, HDL-cholesterol, apo A-I, and ox-LDL with gender, presence of HCV co-infection, lipodystrophy, CD4+ cell count, and viral load

Characteristic	PON1 activity (U/L)	*P*	PON1 concentration (mg/L)	*P*	HDL-cholesterol (mmol/L)	*P*	Apo A-I (g/L)	*P*	ox-LDL (U/L)	*P*
Gender		0.67		0.17		0.10		0.04		0.29
Male	334.5 (166.3)		130.6 (149.8)		1.22 (0.44)		1.34 (0.30)		84.82 (31.90)	
Female	321.2 (140.9)		156.4 (157.9)		1.17 (0.51)		1.43 (0.33)		86.58 (36.36)	
HCV co-infection		<0.001		0.69		0.57		0.28		<0.001
Yes	312.5 (157.2)		149.5 (178.2)		1.18 (0.39)		1.35 (0.30)		77.06 (30.14)	
No	375.2 (157.1)		123.4 (103.3)		1.20 (0.62)		1.39 (0.34)		100.65 (37.9)	
Lipodystrophy		0.22		0.57		0.02		0.47		0.001
Yes	374.6 (181.4)		134.4 (169.4)		1.02 (0.37)		1.33 (0.24)		101.00 (40.00)	
No	333.0 (152.3)		135.4 (146.6)		1.25 (0.53)		1.37 (0.34)		81.63 (31.84)	
Viral load (copies/mL)		0.49		0.004		0.001		0.06		0.52
<200	329.7 (153.5)		108.9 (88.3)		1.23 (0.56)		1.40 (0.32)		83.78 (37.32)	
≥200	345.0 (164.4)		178.5 (209.2)		1.12 (0.36)		1.32 (0.27)		86.79 (30.16)	
CD4+ T lymphocytes (cells/mm^3)		0.22		0.94		0.06		0.30		0.79
<200	335.1 (157.8)		159.6 (129.9)		1.14 (0.39)		1.36 (0.31)		83.80 (35.50)	
200–500	357.6 (147.3)		128.2 (177.7)		1.15 (0.42)		1.33 (0.28)		86.08 (27.88)	
>500	317.2 (152.89		131.4 (156.7)		1.26 (0.63)		1.41 (0.35)		88.30 (38.10)	

Results are presented as means and S.D. in parenthesis.

Polymorphisms of *PON1* did not show any statistically significant association with HDL-cholesterol, apo A-I, or ox-LDL concentrations, either in the control group or in the HIV-infected patients (data not shown).

3.3. Clinical and immunological factors influencing PON1 status, HDL-cholesterol, apo A-I and ox-LDL

The results are summarised in Table 4. Co-infection with HCV was associated with a significantly lower PON1 activity ($P < 0.001$), and ox-LDL concentration ($P < 0.001$). There were no significant differences regarding PON1, HDL or apo A-I concentrations.

Patients with an active viral replication presented with higher PON1 concentrations ($P = 0.004$) and lower HDL-cholesterol levels ($P = 0.001$); no significant differences were found in PON1 activity between these two groups. The three groups stratified according to the CD4+ T lymphocyte count did not present with any significant differences in PON1 status, HDL-cholesterol, apo A-I or ox-LDL levels.

The presence of lipodystrophy was associated with lower HDL-cholesterol concentrations ($P = 0.024$) and higher ox-LDL levels ($P = 0.001$). CD4+ T and CD8+ T lymphocyte counts showed weak negative correlations with PON1 concentration ($\rho = -0.150$, $P = 0.045$, and $\rho = -0.168$, $P = 0.044$, respectively). Serum β-2-microglobulin concentration was negatively related to apo A-I concentration ($\rho = -0.219$, $P = 0.001$), cholesterol-HDL ($\rho = -0.174$; $P = 0.012$) and PON1 activity ($\rho = -0.148$; $P = 0.037$). β-2-Microglobulin showed a positive relationship with PON1 concentration ($\rho = 0.264$; $P = 0.001$).

Patients treated with non-nucleoside retrotranscriptase inhibitor-based combination therapy showed no significant differences in PON1 status compared to the patients treated with protease inhibitors, nor with respect to patients receiving other types of treatment.

3.4. Multivariate analysis for PON1 activity and concentration in HIV-patients

Serum PON1 activity showed significant, and independent, relationships with CD4+ T lymphocyte cell counts [$B = 0.062$ (0–0.123); $P = 0.049$], apo A-I concentration [$B = 106.75$ (42.84–170.66); $P = 0.001$], $PON1_{192}$ [$B = 180.0$ (150.6–209.4); $P < 0.001$], and $PON1\_{107}$ [$B = -57.2$ (-81.8 to (-32.6)); $P < 0.001$] genotypes. Serum PON1 concentration showed a strong significant relationship only with β-2-microglobulin concentration [$B = 75.19$ (50.32–100.06); $P < 0.001$].

4. Discussion

PON1 is an esterase/lactonase with a broad spectrum of possible substrates [11,22] and its physiological function is not, as yet, completely understood. However, a considerable body of evidence suggests that, among other roles, PON1 may hydrolyse oxidised lipid peroxides from low-density lipoproteins (LDL) and HDL [8,10,23–25] and, thus, possess antioxidant and anti-inflammatory properties. PON1 has been studied in relation to diseases involving oxidative stress, including cardiovascular disease [15], systemic lupus erythematosus [26], rheumatoid arthritis [27], renal insufficiency [28], and chronic liver damage [14].

Viral replication and some clinical manifestations of HIV infection involve an imbalance in reduction–oxidation (redox) status and free radical production [29] and, in this regard, the present study has identified significantly lower serum PON1 activity in HIV-infected patients. It is likely that the HIV-induced oxidised environment could result in an increased binding of the free radicals to the PON1 resulting in a less active PON1 in the circulation; similar to the reports of oxidised lipids reacting with a free –SH group at PON's cysteine-284 site leading to inactivation of the enzyme [30]. Another reason for this decrease could be the lower concentrations of HDL-cholesterol and apo A-I that we observed in the patient group. A third reason could be an inhibitory effect of the medical treatment on PON1 activity, since it has been recently reported that some antibiotics inhibit PON1 *in vitro* [31]. Differences in PON1 activity between patients and controls are not due to variations in allele or genotype frequency distribution of *PON1* gene polymorphisms since they were observed to be similar in both groups in our study, and to those for the general Caucasian population [17]. In this respect, HIV infection differs from HCV infection in which we observed a slight increase in the frequency of patients carrying the $PON1_{192}$ RR allele [32]. In the present study we found an increased serum PON1 concentration associated with the HIV infection. This finding may, initially, appear contradictory but the findings agree with a previous observation of decreased PON1 activity and increased concentration in patients with chronic liver impairment [20]. As an explanation, we would hypothesise that the hepatic up-regulation of PON1 synthesis is an attempt to counteract the increased oxidative environment observed in some chronic inflammatory diseases, HIV infection included.

That changes in PON1 status play a role in the course of HIV infection is an area that is worthy of further investigation. Changes in the relative proportions of some HDL components, such as apo A-I and PON1, are observed in infectious diseases [33–35]. Apo A-I inhibits those steps in HIV infection involving membrane fusion [36] and, as such, higher apo A-I concentrations would be associated with lower viral infectivity. We hypothesise that PON1 may also play an anti-infective role, since this enzyme increases the cholesterol efflux from the cell and the binding of the HDL particle to its receptor (ABCA1) [34]. It is of note that membrane metabolism is modulated by the efflux of cholesterol to the HDL particle (i.e. HDL particles deplete cholesterol from the cell membrane) and that this phenomenon would influence HIV replication since the virus needs the cholesterol rafts of the cell membrane to perform

the final viral assembly [4,37,38]. Therefore, a possibility exists that PON1 status could play a role in pre-empting HIV replication and allowing the normal cholesterol turnover from the cell membranes to proceed.

An important issue that needs to be resolved is whether the replication of the HIV1 virus itself would be related to PON1 status. We did not find any significant differences in PON1 activity in relation to the presence of a negative viral load but, interestingly, there was a significant increase in PON1 concentration in the patients with active viral replication. In addition, we also observed a positive association between serum PON1 and β-2-microglobulin concentration; the latter being an effective marker of HIV infection activity [18].

Recently, the study of the relationship between HDL and the course of AIDS has attracted a great deal of attention from the scientific community. The adaptation of this lipoprotein to a chronic inflammatory and an oxidant state, together with its relationships to HIV-related metabolic disturbances, are areas that are receiving greater attention because, with the new treatments becoming available, these patients have longer survival times and, as such, other physiological perturbations are becoming increasingly relevant [39–41]. The results of the present study suggest that PON1 status may play a role in rectifying these derangements. This concept warrants further research since it represents an aspect that may engender beneficial consequences for these patients.

Acknowledgments

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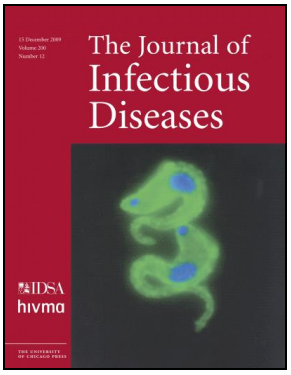
ESTUDI 2

Paraoxonase-1 gene haplotypes are related to metabolic disturbances, atherosclerosis and immunologic outcome in HIV-infected patients.

En premsa.



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MAJOR ARTICLE

Association of Paraoxonase-1 Gene Haplotypes with the Immunologic Outcome of and Metabolic Disturbances and Atherosclerosis in HIV-Infected Patients

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Background. Oxidative stress is associated with human immunodeficiency virus (HIV) infection. Paraoxonase-1 (PON1) is an antioxidant enzyme that is bound to high-density lipoproteins (HDLs). We evaluated whether *PON1* gene haplotypes influence the metabolic disturbances, presence of subclinical atherosclerosis, and virologic outcome associated with the infection.

Methods. DNA from blood samples collected from 234 HIV-infected patients and 633 healthy control subjects had single-nucleotide polymorphisms of $PON1_{192}$, $PON1_{55}$, $PON1_{-162}$, $PON1_{-832}$, $PON1_{-909}$, $PON1_{-1076}$, and $PON1_{-1741}$ analyzed using the Iplex Gold MassArray method. Subsequently, the influence of these single-nucleotide polymorphisms on measured biochemical and clinical variables was assessed.

Results. We observed significant differences in the haplotype distribution between the control subjects and the HIV-infected patients. Haplotype H10 (GTCCGTC) was more prevalent in the HIV-infected patients (6.41% vs 0.64%; $P < 2.502$), and haplotype H5 (GACCGTC) was less prevalent in HIV-infected patients (27.7% vs 42.9%; $P = .001$). In HIV-infected patients, haplotype H7 (AATTCCT) was associated with better $CD4^+$ cell count recovery, higher levels of HDL cholesterol ($P = .048$) and apolipoprotein A-I ($P = .019$), lower levels of triglycerides ($P = .004$), and lower rates of subclinical arteriosclerosis ($P < .001$).

Conclusions. *PON1* haplotypes segregate with HIV infection, HDL metabolism, the presence of subclinical atherosclerosis, and $CD4^+$ cell recovery after treatment.

The spread of human immunodeficiency virus type 1 (HIV-1) infection continues to increase, whereas the survival of HIV-1–infected patients is considerably extended by new and more-effective antiretroviral therapy. Hence, it is likely that long-term consequences of treatment and infection will become increasingly common. These consequences involve not only the immunocompromised status of the patient but also metabolic derangements, including lipoprotein disorders that may lead to cardiovascular disease [1]. One of these problems is increased oxidative stress resulting from either the infection itself or the secondary effects of treatments [2]. Paraoxonase-1 (PON1) is an enzyme with antioxidant properties. PON1 is an esterase and lactonase that catalyzes the hydrolysis of oxidized phospholipids and lipophylic lactones [3]. In the general population and in patients with diabetes, PON1 preserves high-density lipoproteins (HDLs) and low-density lipoproteins (LDLs) from peroxidation and, as such, has been associated with a protective role against the development of atherosclerosis [4–8]. *PON1* knockout mice have increased macrophage oxidative stress and are more susceptible to atherosclerosis [9],

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Table 1. General Characteristics of 234 Human Immunodeficiency Virus (HIV)–Infected Patients

Characteristic (no. of patients)[a]	Value
Age, mean ± SD, years (n = 234)	38.7 ± 6.8
Sex, male (n = 234)	162 (69.2)
Conventional risk factor for cardiovascular disease (n = 234)	
Current smoker	184 (78.6)
Hypertension	21 (9.0)
Abnormal fasting glucose level	20 (8.5)
Body mass index, mean ± SD, kg/m^2	23.1 ± 3.2
Dyslipidemia	85 (36.3)
Risk factors for HIV infection (n = 234)	
Intravenous drug use	133 (56.8)
Male homosexual contact	29 (12.3)
Heterosexual contact	68 (29.1)
Time since HIV diagnosis, mean ± SD, years (n = 234)	5.4 ± 3.3
Baseline CD4$^+$ cell count, mean ± SD, U/mm^3 (n = 185)	359.9 ± 297.3
Viral load <200 copies/mL (n = 234)	92 (39.3)
AIDS-related disease (n = 234)	77 (32.9)
HCV coinfection (n = 234)	142 (60.6)
Lipodystrophy (n = 234)	51 (21.8)
Duration of previous ART received, mean ± SD, months (n = 234)	
Nucleoside analogues	103.3 ± 62.7
Protease inhibitor	30.2 ± 27.3
Nonnucleoside analogues	8.1 ± 10.4
Treated with statins (n = 234)	6 (2.6)
Treated with fibrates (n = 234)	20 (8.5)
Lipid profile	
Cholesterol, mean ± SD, mmol/L (n = 186)	4.9 ± 1.3
HDL cholesterol, mean ± SD, mmol/L (n = 202)	1.2 ± .5
LDL cholesterol, mean ± SD, mmol/L (n = 171)	2.8 ± 1.0
Triglycerides, mean ± SD, mmol/L (n = 184)	2.3 ± 2.1
Apolipoprotein A-I, mean ± SD, g/L (n = 146)	1.4 ± .3
Apolipoprotein A-II, mean ± SD, g/L (n = 196)	.33 ± .06

NOTE. Quantitative variables are expressed as the mean value ± standard deviation. Qualitative variables are expressed as the no. (%) of patients. ART, antiretroviral therapy; HCV, hepatitis C virus; HDL, high-density lipoprotein; HIV, human immunodeficiency virus; LDL, low-density lipoprotein; SD, standard deviation.

[a] No. of patients for whom information was available.

and human *PON1* transgenic mice have decreased atherosclerosis formation and lipoprotein oxidation [10]. PON1 attenuates in vitro production of monocyte chemoattractant protein–1 (MCP-1) by monocytes. MCP-1 is a proinflammatory chemokine that is involved in the initial step of formation of the atheromatous plaque [11]. Previous studies from our group demonstrated an increased plasma MCP-1 concentration in HIV-infected patients and an association between the polymorphisms in the *MCP-1*$_{-2518}$ allele and the presence of subclinical atherosclerosis [12]. We have also reported elsewhere [13] that PON1 status is influenced by the course of HIV infection and results in a decrease in PON1 activity.

The hypothesis of the present study is that the relationship between oxidative stress, HIV infection, and atherosclerosis is such that *PON1* gene polymorphisms could be associated with the metabolic disturbances associated with the infection, as well as with the immunologic, virologic, and clinical course of this disease.

METHODS

Study population and design. In an initial study, we performed case-control comparisons to assess the differences in genotype distributions of the *PON1* genes in HIV-infected patients, compared with differences in the *PON1* gene distributions noted in the general population. The study participants who were used as the control group enrolled in a population-based study conducted in our area. Details of this study have

Table 2. Differences between the Haplotypes Distribution in the Control Subjects and the Human Immunodeficiency Virus (HIV)–Infected Patients

Haplotype (sequence)	Control subjects, no. (%) (n = 633)	HIV-infected patients, no. (%) (n = 234)	P
H1[a] (AACCGTC)	111 (17.5)	55 (23.5)	.054
H2[a] (ATCCGTC)	563 (88.9)	199 (85.04)	.760
H3[a] (GACTCTT)	57 (9.0)	16 (6.8)	.419
H4[a] (AACCCTT)	96 (15.1)	41 (17.5)	.368
H5[a] (GACCGTC)	272 (42.9)	65 (27.7)	.001
H6 (ATTTCCT)	11 (1.7)	3 (1.3)	1.000
H7* (AATTCCT)	240 (37.9)	81 (34.61)	.631
H8* (GACCCTT)	80 (12.6)	31 (13.2)	.740
H9 (ATCCGTT)	1 (.15)	0 (0)	1.000
H10* (GTCCGTC)	4 (.63)	15 (6.41)	<2.502
H11* (AATTCCC)	47 (7.42)	15 (6.41)	.771
H12* (GATTCCT)	48 (7.58)	21 (8.97)	.488
H13 (GACCTGG)	4 (.63)	0 (0)	.578
H14 (ATCCCTT)	32 (5.05)	8 (3.41)	.469
H15 (GTTCCTT)	1 (.15)	0 (0)	1.000
H16 (AACTCTT)	9 (1.42)	9 (3.84)	.031
H17 (GTCCCTT)	12 (1.89)	9 (3.84)	.131
H18 (AACCCTC)	4 (.63)	3 (1.28)	.390
H19 (AACTCCT)	1 (.15)	0 (0)	1.000
H20 (ATTCCTT)	1 (.15)	0 (0)	1.000
H21 (ATTTCCC)	1 (.15)	0 (0)	1.000
H22 (GTTTCCT)	1 (.15)	0 (0)	.265
H23 (GATCCTT)	1 (.15)	0 (0)	.265
H24 (AATCCTT)	1 (.15)	0 (0)	.265
H25 (GACCCCT)	1 (.15)	0 (0)	.265

[a] Haplotype with a frequency >5%.

been published elsewhere [14]. In brief, the 633 participants were ostensibly healthy individuals (339 women and 294 men; q11 mean age, 45 years [range, 18–81 years]) of white ethnic origin who were from the Mediterranean region of Catalunya. The 234 HIV-infected patients who were studied (72 women and 162 men; mean age, 39 years [range, 20–66 years]) were among those attending our outpatient AIDS clinic and were of the same ethnic origin as the control participants in the study. The only exclusion criterion was age <18 years.

q12 Expanding on the initial study, we performed a case-control assessment of the HIV-infected patients receiving antiretroviral q13 treatment. The purpose of the assessment was to evaluate any associations of *PON1* gene haplotypes with the metabolic, immunologic, and virologic variables measured. In this second study, we defined as "cases" those HIV-infected patients who q14 had lipodystrophy, metabolic disturbances, dyslipidemia (as defined by the National Cholesterol Education Program Adult Treatment Panel III as a total cholesterol level >5.0 mmol/L or an LDL cholesterol level >3.0 mmol/L; an HDL cholesterol <1.0 mmol/L in men and <1.2 in women; or a triglyceride level >1.7mmol/L), a positive cardiovascular disease risk (as assessed by the Framingham risk score), and the presence of atherosclerosis, as determined by measurement of the intima-media thickness (IMT) in the carotid artery. Although IMT is a con- q15 tinuous variable, and although we have reported the data in this form, we also defined subclinical atherosclerosis as (1) a categorical (dichotomized) variable when the IMT was >0.8 mm or (2) the presence of an atheromatous plaque in the analyzed zones of the arteries.

We used previously established definitions of cases and con- q16 trols in association with the magnitude of the increase in the $CD4^+$ cell count—that is, we reassigned the HIV-infected patients as cases or controls on the basis of their response or lack of response, respectively, to anti-HIV treatment. Patients who q17 did not have a $CD4^+$ cell count increase of >50 cells/mm^3 after 12 months of treatment follow-up were considered to be "nonresponders" [15]. In terms of virologic variables, we considered cases to be those patients who experienced a rebound in the viral load during the course of the 12-month observational period of the study. Patients who abandoned treatment and those who completed the 12-month observational period with a negative viral load were censored from the present statistical analyses.

All the participants provided fully informed consent. The data were coded to ensure anonymity. The study was approved by the ethics committee of the Hospital Universitari de Sant Joan de Reus. All procedures were performed according to the principles of the Declaration of Helsinki and Good Clinical Practice.

Biochemical measurements. A sample of fasting venous blood was obtained during the clinical examination. The plasma viral load was measured using the Cobas TaqMan HIV-1 assay (Roche), and the $CD4^+$ T cell count was determined using flow cytometry (Beckman-Coulter). HDL cholesterol levels were analyzed by a homogeneous method (Beckman-Coulter). LDL cholesterol levels were calculated using the Friedewald formula [16]. Serum total cholesterol and triglyceride concentrations were measured by standard methods (Beckman-Coulter). Serum PON1 esterase activity was measured as the rate of hydrolysis of paraoxon at 410 nm and 37°C in 0.05 mmol/L glycine buffer (pH 10.5) with 1 mmol/L calcium chloride [17]. Activities were expressed as the number of units per liter, where q18 1 unit equals 1 micromole of paraoxon hydrolyzed per minute. Serum PON1 lactonase activity was measured in an assay reagent containing 1 mmol/L calcium chloride, 0.25 mmol/L 5-(thiobutyl)-butyrolactone (TBBL), and 0.5 mmol/L 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 0.05 mmol/L Tris-HCL buffer (pH 8.0). The increase in absorbance was monitored at 412 nm [18, 19]. Activities were expressed as the number of units per liter, where 1 unit equals 1 millimole of TBBL hy-

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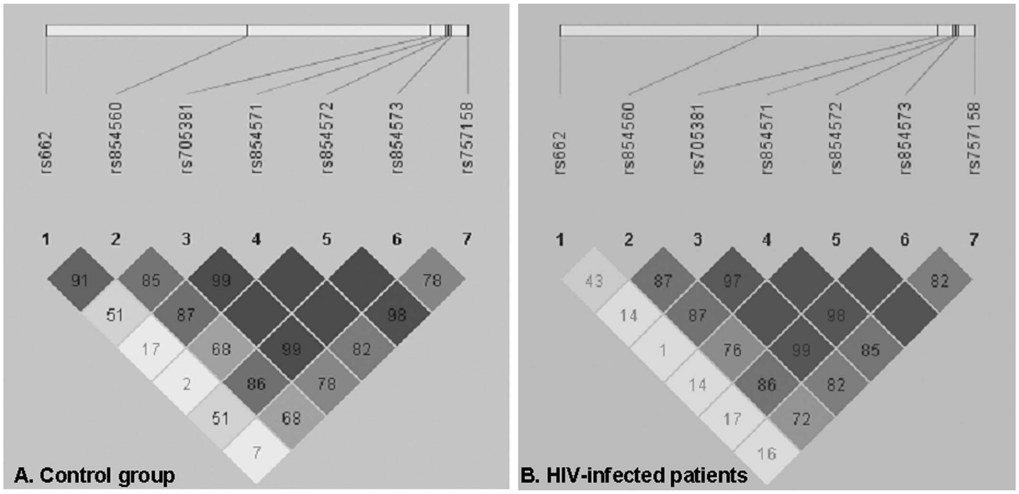

Figure 1. Linkage disequilibrium structure of the *PON1* gene in control subjects *(A)* and human immunodeficiency virus (HIV)–infected patients *(B)*. Linkage disequilibrium structure of 7 common single-nucleotide polymorphisms (SNPs) labeled by their RS numbers vertically. Note that SNP number 1 is $PON1_{192}$, followed by $PON1_{55}$, $PON1_{-162}$, $PON1_{-832}$, $PON1_{-909}$, $PON1_{-1076}$, and $PON1_{-1741}$. Each square denotes the strength and significance of linkage disequilibrium between pairs of markers in the region. Red denotes no (or minimal) evidence of historical recombination. The numbers of squares denote 100XD′ (a statistical measure of linkage disequilibrium), with missing values denoting a result of 100.

drolyzed per minute. The serum PON1 concentration was determined by enzyme-linked immunosorbent assay (ELISA) performed using an antibody raised against a peptide derived from the sequence of mature PON1 [6]. The serum concentration of oxidized LDL was measured by ELISA (Mercodia). Serum apolipoprotein (apo) A-I and A-II concentrations were determined by immunoturbidimetry (Beckman-Coulter and Dialab Gmbh, respectively). The serum concentration of C-reactive protein (CRP) was measured using a high-sensitivity method (Beckman-Coulter). The plasma concentration of MCP-1 was measured by ELISA (Human MCP-1 ELISA Development Kit; Prepotech).

Genotyping. Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set; Gentra Systems). $PON1_{192}$, $PON1_{55}$, $PON1_{-162}$, $PON1_{-832}$, $PON1_{-909}$, $PON1_{-1076}$, $PON1_{-1741}$, and $MCP\text{-}1_{-2518}$ single-nucleotide polymorphisms (SNPs) were analyzed using the Iplex Gold MassArray method (Sequenom) at the Spanish National Genotyping Center (Centro Nacional de Genotipado of the Universitat Pompeu Fabra, Barcelona, Spain).

Arterial IMT measurement. We performed carotid and femoral ultrasound measurements for 183 HIV-infected patients. By use of an identical protocol, these measurements were q19 conducted by the same investigators who performed a previously published study [12], all of whom were blinded with respect to the results of the other variables studied. We used a GE Logiq 700 with an ultrasound probe of 7–10 MHz. We identified 3 segments in the carotid arteries on which to conduct the measurements: the common carotid artery (1 cm proximal to the bifurcation), the carotid bulb (in the bifurcation), and the internal carotid artery (1 cm distal to the bifurcation). We evaluated the common femoral artery 1 cm proximal to the bifurcation. The far-wall IMT images were obtained and q20 digitized for each participant.

Statistical analysis. We used the χ^2 test to assess the degree of association between categorical variables. An analysis of variance (ANOVA) test or Student's *t* test was used for continuous variables that followed a normal distribution. The Mann-Whitney *U* test and the Wilcoxon rank-sum test were used for nonparametric variables. The results are presented as the mean value ± standard deviation (SD), for parametric variables, and the median value (range), for nonparametric variables. SNPs were tested for Hardy-Weinberg equilibrium by use of Haploview software (version 4.0) [20]. Estimates of linkage disequilibrium between SNPs were calculated using D′ and r^2. Haplotype estimations were performed using PHASE software q22 [21] with default settings and the SNPator package [22]. Linear or logistic regression models were used to identify the haplotypes that predicted the dependent variables adjusted for potential confounding factors, such as age, sex, dyslipidemia, smoking habit, hypertension, fasting glucose, body mass index, hepatitis C virus coinfection, lipid-lowering treatment, duration of each antiretroviral treatment scheme, and basal $CD4^+$ cell count. The Kaplan-Meyer hazard model was used to determine q24 the association between the haplotypes and the time to an undetectable viral load at a 95% confidence interval (CI). All statistical analyses were performed using the SPSS statistical package (version 15.0; SPSS).

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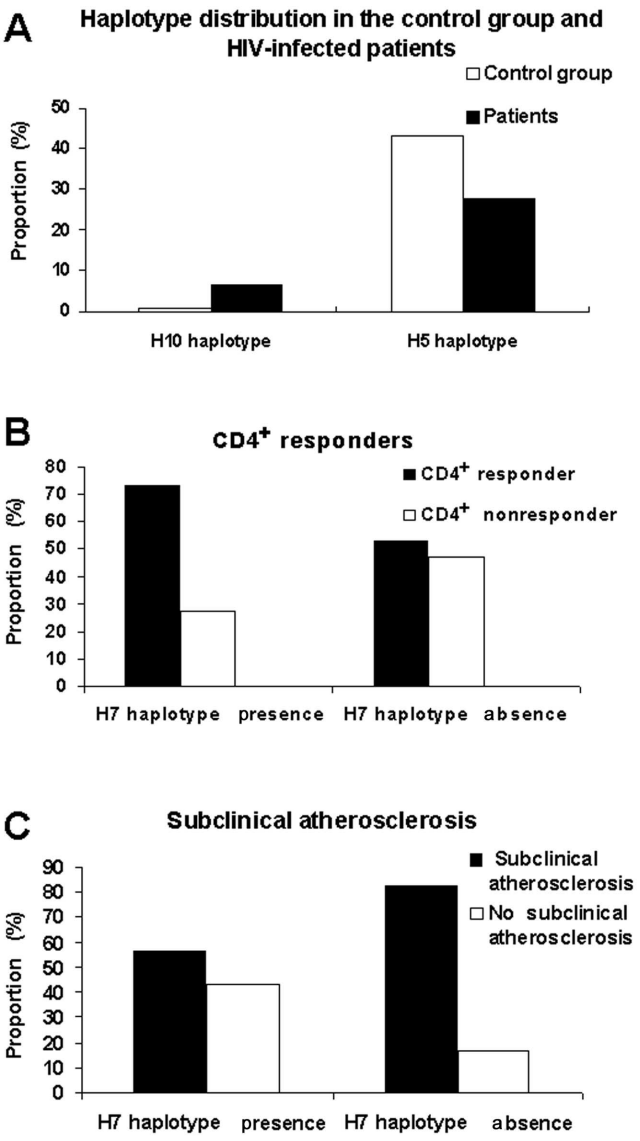

Figure 2. *A,* Differences between the haplotype distributions in control subjects and human immunodeficiency virus (HIV)–infected patients. *B,* Differences in the proportion of HIV-infected patients with a $CD4^+$ cell response in relation to the presence of absence of haplotype H7. *C,* Differences in the proportion of HIV-infected patients with subclinical atherosclerosis in relation to the presence or absence of haplotype H7.

RESULTS

Genotype distributions in control subjects and HIV-infected patients. The general characteristics of the patients are summarized in Table 1. We obtained 25 different haplotypes corresponding to the SNP mutations $PON1_{192}$, $PON1_{55}$, $PON1_{-162}$, $PON1_{-832}$, $PON1_{-909}$, $PON1_{-1076}$, and $PON1_{-1741}$ (Table 2). We observed strong linkage disequilibrium between all the SNPs in HIV-infected patients ($D' = 43$), except for the SNP between the $PON1_{192}$ and $PON1_{55}$ polymorphisms (Figure 1). We observed significant differences in the distributions of haplotype H10 (odds ratio [OR], 10.6 [95% CI, 3.52–32.2]; $P < 2.502$) and haplotype H5 (OR, 0.61 [95% CI, 0.46–0.83]; $P = .001$) between control subjects and HIV-infected patients (Figure 2*A*).

Association of* PON1 *haplotypes with the immunologic and virologic outcomes in HIV-infected patients. In a bivariate analysis, we found significant differences in the $CD4^+$ cell counts of HIV-infected patients segregated according to haplotype. Patients carrying the H7 haplotype had higher basal $CD4^+$ cell counts (Figure 2*B* and Table 3). After segregating patients according to $CD4^+$ cell count, we also observed a greater number of responders among patients carrying the H7 haplotype than among patients who did not carry this haplotype (72.9% vs 52.7%; $P = .017$).

The probability of maintaining an undetectable viral load while receiving antiretroviral treatment was also significantly associated with *PON1* gene haplotypes. Carrying haplotypes H10 and H5 was associated with a higher probability of maintaining viral suppression ($P = .047$ and $P = .022$, respectively).

Association of* PON1 *haplotypes with the presence of lipodystrophy, metabolic disturbances, cardiovascular disease risk, and dyslipidemia. We did not observe (by use of bivariate analysis) any association between *PON1* haplotypes and lipodystrophy, metabolic disturbances, or the 10-year risk of cardiovascular disease, as assessed by the Framingham risk score. However, we did find an association between dyslipidemia and the H7 and H4 haplotypes ($P = .025$ and $P = .006$, respectively). Patients carrying the H7 haplotype had lower serum triglyceride concentrations, higher HDL cholesterol levels, and higher apo A-I and apo A-II concentrations (Table 3).

HIV-infected patients carrying the H4 haplotype also had lower serum triglyceride levels (mean ± SD, 2.16 ± 2.05 mmol/L vs 3.09 ± 2.35 mmol/L; $P = .042$) and higher HDL cholesterol levels (1.23 ± 0.53 mmol/L vs 0.97 ± 0.25 mmol/L; $P = .012$). In a linear logistic regression model, HDL cholesterol levels (as a dependent variable) were predicted by triglycerides ($\beta = -.075$ [95% CI, −.107 to −.044]; $P < .001$) and the duration of nonnucleoside antiretroviral treatment ($\beta = .009$ [95% CI, .004 to .015]; $P < .001$). In a linear regression model, serum apo A-I concentrations were predicted by the H7 haplotype ($\beta = .176$ [95% CI, .070–.283]; $P = .008$), the duration of antiretroviral treatment ($\beta = .008$ [95% CI, .003–.0012]; $P < .001$), and hepatitis C virus coinfection ($\beta = -.106$ [95% CI, −.119 to .012]; $P = .028$).

Association of PON1 haplotypes with the presence of subclinical atherosclerosis in HIV-infected patients. We found an association between the presence of subclinical atherosclerosis and the H7 haplotype (Figure 2*C*). Patients who did not carry this haplotype had higher rates of subclinical atherosclerosis

Table 3. Differences in the Lipid Profile, Inflammatory and Oxidative Markers, PON1 Levels, and CD4⁺ Cell Counts in Human Immunodeficiency Virus (HIV)–Infected Patients Segregated with Respect to Presence of the H7 Haplotype

Laboratory value or characteristic, H7 haplotype carrier	Patients, no.	Value[a]	*P*
Cholesterol level, mmol/L			
Yes	49	5.05 ± 1.45	.260
No	137	4.89 ± .94	.260
HDL cholesterol level, mmol/L			
Yes	56	1.28 ± .45	.048
No	146	1.14 ± .44	
LDL cholesterol level, mmol/L			
Yes	47	2.81 ± .79	.832
No	124	2.77 ± 1.07	
Apolipoprotein A-I level, g/L			
Yes	53	1.49 ± .28	.019
No	93	1.35 ± 7.0	
Apolipoprotein A-II level, g/L			
Yes	51	.35 ± .07	.030
No	145	.32 ± .06	
Triglycerides level, mmol/L			
Yes	49	1.36 (.6–5.6)	<.001
No	135	2.77 ± 1.07	
PON1 esterase activity, U/L			
Yes	57	258.6 (115.1–667.0)	.090
No	153	306.8 (75.9–818.1)	
PON1 lactonase activity, U/L			
Yes	56	5.10 ± 1.58	.702
No	158	5.19 ± 1.69	
PON1 level, U/L			
Yes	56	126.1 ± 107.6	.509
No	151	140.9 ± 166.1	
Ox-LDL level, U/L			
Yes	57	82.14 ± 38.3	.329
No	158	87.4 ± 33.01	
CRP level, mg/L			
Yes	57	4.48 ± 7.97	.528
No	152	3.90 ± 4.07	
MCP-1 level, ng/L			
Yes	47	73.65 ± 41.65	.718
No	125	76.48 ± 47.20	
Basal CD4⁺ cell count, cells/mm^3			
Yes	49	425.9 ± 320.9	.035
No	136	318.1 ± 282.5	
Age, years			
Yes	49	37.2 ± 7.1	.056
No	136	39.7 ± 7.3	

NOTE. CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MCP-1, monocyte chemoattractant protein–1; ox-LDL, oxidized LDL.

[a] Data are expressed as the mean ± standard deviation, for variables with parametric distributions, and as the median (range), for variables with nonparametric distributions.

rosis (83.3% vs 16.7%; $P < .001$). In the logistic regression model, the variables that predicted presence of atherosclerosis were age ($\beta = 1.261$ [95% CI, 1.136–1.401]; $P < .001$), the *MCP-1 −2518G* allele ($\beta = .162$ [95% CI, .056–.470]; $P = .001$), and the H7 haplotype ($\beta = .381$ [95% CI, .1510–.961]; $P = .041$).

The absence of the H7 haplotype was also associated with an increased IMT as a continuous variable (mean ± SD, 0.78 ± 0.19 vs 0.70 ± 0.16 mm) in patients carrying the H7 haplotype ($P = .010$). In the linear regression model, the variables that predicted the IMT values in the carotid arteries were age ($\beta = .010$ [95% CI, .006–.014; $P < .001$), the duration of antiretroviral treatment ($\beta = .004$ [95% CI, .001–.007]; $P = .005$), and the H7 haplotype ($\beta = -.075$ [95% CI, −1.141 to .010]; $P = .026$).

We did not observe any significant association between the serum PON1 activity or concentration and the immunologic, virologic, metabolic, or clinical variables analyzed.

DISCUSSION

The results of the present study show significant differences between control subjects and HIV-infected patients with respect to the genetic distribution of 2 *PON1* gene haplotypes. The H10 haplotype is more prevalent in HIV-infected patients than in control subjects, although the small number of participants carrying this haplotype makes this conclusion very preliminary. The absence of the H10 haplotype is also associated with a higher probability of maintaining a negative viral load. As such, we can suggest that this haplotype is more prevalent in HIV-infected patients because it is associated with increased patient survival. The H5 haplotype is significantly less prevalent in HIV-infected patients than in control subjects, and its absence is also associated with a higher probability of maintaining viral suppression. Perhaps patients with this genotypic background show less capability of controlling viral replication when receiving antiretroviral treatment and may be less protected in the first stages of HIV transmission.

The strong linkage disequilibrium observed between the SNPs of *PON1* also allowed us to detect candidate SNPs with functional and clinical implications that warrant further investigation. $PON1_{192}$ codes for isoenzymes that show different functional antioxidant activity, as has been reported elsewhere [5, 23]. The H10 and H5 haplotypes contain the R allele at position 192; the R allele is the allele that is less effective in the protection against lipid peroxidation [7]. The H7 haplotype appears to have an important role with respect to the lipid profile, the presence of atherosclerosis, and the $CD4^+$ cell count. We observed that patients who carried this haplotype had higher levels of HDL cholesterol and apo A-I, as well as lower triglyceride levels. The H7 haplotype was also associated with lower rates of subclinical atherosclerosis. In the multivariate regression model, not carrying the haplotype predicted the presence of atherosclerosis, after adjustment for other classical cardiovascular disease risk factors. Also in the regression model, the adjusted apo A-I levels, but not the triglyceride levels, were predicted by the H7 haplotype. That apo A-I and HDL levels are influenced by the *PON1* polymorphisms can be hypothesized to be an effect related to conformational changes in the PON1 isoform which confers a more stable binding to apo A-I [24]. The H7 haplotype carries the Q allele at the $PON1_{192}$ position, and this allele is known to confer better antioxidant properties and better protection against the development of atherosclerosis [5].

It is of note that the H7 haplotype was also associated with higher basal $CD4^+$ cell counts and better $CD4^+$ cell recovery with treatment. The basal $CD4^+$ cell count is an independent risk factor for atherosclerosis in HIV-infected patients [25, 26]. As such, it is possible that the *PON1* genotypic background modulates the lipid profile and also confers a better oxidative status, which, in turn, lowers $CD4^+$ cell apoptosis and, consequently, lowers the predisposition to atherosclerosis related to the proinflammatory and prooxidative status of this patient population.

A caveat of the present study is that, despite a clear association between *PON1* gene haplotypes and severe complications of HIV infection, we did not observe any significant association between these derangements and circulating levels of PON1, with respect to either the activity or the concentration of the enzyme. The explanation for this phenomenon cannot be ascertained from the present study, but a possible underlying mechanism could be related to the aforementioned observation—that is, the $PON1_{192}$ polymorphism may influence PON1 binding to apo A-I and HDL, causing differences in enzyme stability and modifying HDL function independently of PON1 levels [24]. Our results showing that patients who carry the H7 haplotype (which contains the $PON1_{192}$ polymorphism) have higher HDL cholesterol and apo A-I concentrations would tend to support this hypothesis. Another limitation of the present study is its cross-sectional design, which makes any conclusions about associations with disease progression and pathogenesis very preliminary. Prospective, longitudinal studies investigating the influence of *PON1* haplotypes on seroconversion and patient survival would be necessary to describe more clearly the role of these haplotypes in disease progression.

In conclusion, *PON1* haplotypes are associated with HDL and apo A1 levels, the presence of atherosclerosis, immunologic status, and virologic outcome after treatment. The clinical implication is that novel therapeutic strategies may need to take into account the *PON1* genotypic background of the patient, especially if the therapeutic option is to modulate serum PON1 activity and HDL or apoA-I levels [27, 28]. Our results suggest that the PON1-HDL complex may play a role in the homeo-

stasis of atherosclerosis and HIV infection (ie, the HDL part of the complex is involved in reverse cholesterol transport and catabolism, whereas the PON1 part modulates inflammation, oxidative status, and the immunologic system). This concept warrants further research, because it is an aspect that may provide beneficial consequences for these patients for whom effective treatment options with less toxic effects currently are not very extensive.

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ESTUDI 3

Nonconcordance between subclinical atherosclerosis and the calculated Framingham risk score in HIV-infected patients: relationships with serum markers of oxidation and inflammation.

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HIV Medicine (2009)

ORIGINAL RESEARCH

Nonconcordance between subclinical atherosclerosis and the calculated Framingham risk score in HIV-infected patients: relationships with serum markers of oxidation and inflammation

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Objectives
HIV-infected patients show an increased cardiovascular disease (CVD) risk resulting, essentially, from metabolic disturbances related to chronic infection and antiretroviral treatments. The aims of this study were: (1) to evaluate the agreement between the CVD risk estimated using the Framingham risk score (FRS) and the observed presence of subclinical atherosclerosis in HIV-infected patients; (2) to investigate the relationships between CVD and plasma biomarkers of oxidation and inflammation.

Methods
Atherosclerosis was evaluated in 187 HIV-infected patients by measuring the carotid intima-media thickness (CIMT). CVD risk was estimated using the FRS. We also measured the circulating levels of interleukin-6, monocyte chemoattractant protein-1 (MCP-1) and oxidized low-density lipoprotein (LDL), and paraoxonase-1 activity and concentration.

Results
There was a weak, albeit statistically significant, agreement between FRS and CIMT ($\kappa = 0.229$, $P < 0.001$). A high proportion of patients with an estimated low risk had subclinical atherosclerosis ($n = 66$; 56.4%). In a multivariate analysis, the presence of subclinical atherosclerosis in this subgroup of patients was associated with age [odds ratio (OR) 1.285; 95% confidence interval (CI) 1.084–1.524; $P = 0.004$], body mass index (OR 0.799; 95% CI 0.642–0.994; $P = 0.044$), MCP-1 (OR 1.027; 95% CI 1.004–1.050; $P = 0.020$) and oxidized LDL (OR 1.026; 95% CI 1.001–1.051; $P = 0.041$).

Conclusion
FRS underestimated the presence of subclinical atherosclerosis in HIV-infected patients. The increased CVD risk was related, in part, to the chronic oxidative stress and inflammatory status associated with this patient population.

Keywords: cardiovascular disease, inflammation, lipoproteins, risk factors

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Introduction

Since the advent of effective antiretroviral therapy, HIV infection has become a chronic disease [1]. The life expectancy of HIV-infected patients is progressively improving, but undesirable secondary effects of these treatments and the infection itself are associated with metabolic complications, including dyslipidaemia, insulin resistance, altered body fat distribution and hypertension [2,3]. An increase in atherosclerosis at a relatively young age becomes evident in these patients, probably secondary to the pro-inflammatory and pro-oxidative status of chronic infection exacerbating classical cardiovascular disease (CVD) risk factors, including dyslipidaemia [4–7].

The decision to initiate a treatment to prevent CVD is commonly based on the individual's risk estimation

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generated from equations derived from data for the general population [8–10]. The Framingham risk score (FRS) is the most widely used estimation, and use of the FRS is considered the reference method. In HIV-infected patients, the clinical management of CVD risk is complex because of the wide range of drugs used and their pharmacological interactions. A follow-up of patients within the D:A:D study reported that HIV-infected patients receiving antiretroviral treatment had a risk of developing myocardial infarction that was similar to, or somewhat higher than, that predicted by the FRS [11]. In addition, more recent reports suggest that FRS may underestimate the real CVD risk in HIV-infected patients [12–15].

Although conventional factors undoubtedly play an important role in determining CVD risk in HIV-infected patients, FRS and the other indices do not take into account crucial clinical factors related to chronic HIV infection in these patients, such as their inflammatory and oxidative status. Inflammatory and oxidative parameters, along with surrogate markers of arteriosclerosis, are of considerable interest because they facilitate therapeutic decisions regarding CVD prevention, especially in the clinical management of HIV-infected patients in whom treatment is complex because of multiple drug interactions and opportunistic infections.

The measurement of carotid intima-media thickness (CIMT) has been proposed as a surrogate marker of atherosclerosis and a valuable index of the future appearance of adverse vascular events in the at-risk patient within the general population [16]. We and others have demonstrated an increase in CIMT in HIV-infected patients; these patients also have a faster rate of progression of atherosclerosis [17,18]. This indicates that CIMT is a realistic reflection of arterial lesion status in these patients. Together with CIMT, several biochemical markers of inflammation and oxidation can be analysed to evaluate the early development of arteriosclerosis in HIV-infected patients. C-reactive protein (CRP) is a useful marker of adverse cardiovascular events in the general population [19]. The roles of other plasma constituents are under investigation. For example, interleukin-6 (IL-6) is an inflammatory cytokine that stimulates the liver to increase the production of acute-phase reactants [20]. Monocyte chemotactic protein-1 (MCP-1) is another inflammatory cytokine that enhances the recruitment of monocytes into the subendothelial space, where they differentiate into macrophages and become foam cells. MCP-1 has been shown to be associated with the presence of subclinical atherosclerosis [21] in HIV-infected patients and in those with lipodystrophy [22]. Serum oxidized low-density lipoprotein (oxLDL) has been extensively studied as a marker of oxidative stress. oxLDL and paraoxonase-1 (PON1) are considered to have important functions in the process of atherosclerosis [23]. PON1 is an antioxidant enzyme bound to high-density lipoproteins (HDLs) that attenuates lipid peroxidation and protects against atherosclerosis [24–26].

We previously reported a decrease in PON1 activity and an increase in PON1 concentration in HIV-infected patients [27]. The aim of the present study was to investigate, in a cohort of HIV-infected patients, the relationships among the presence of subclinical atherosclerosis (measured as CIMT), individual CVD risk (estimated using the FRS), and the measured circulating levels of inflammation and oxidation biomarkers.

Methods

Participants and eligibility

The study was observational and cross-sectional. We recruited 187 consecutive HIV-positive patients attending the clinics of the Hospital Universitari de Sant Joan. The exclusion criteria were age <18 years, having an AIDS-related opportunistic disease at the beginning of the study, or having a previous history of clinical CVD. The study was approved by the Ethics Committee of the Hospital and written informed consent was obtained from all the participants in the study.

Clinical and laboratory measurements

A detailed clinical history was taken and a thorough physical examination performed at interview. Anthropometric variables, including body mass index (BMI), gender, age, smoking status and treatment with hypolipidaemic or antiretroviral drugs were recorded. The presence of hypertension or diabetes was defined according to standard international criteria [8]. Lipodystrophy was defined as the presence of body fat changes that could be clearly recognized by the patient and confirmed by the doctor. Body fat changes included subcutaneous lipoatrophy (hollow cheeks, prominent superficial veins on the limbs, or flattening of the buttocks) and central obesity (increased abdominal girth, breast enlargement, or dorsocervical fat pad) [21,22]. A sample of fasting venous blood was obtained during the clinical examination. Serum glucose, cholesterol and triglyceride concentrations were measured by standard methods (Beckman-Coulter, Fullerton, CA, USA). HDL cholesterol was analysed using a homogeneous method (Beckman-Coulter). LDL concentrations were calculated using the Friedewald formula [28]. Serum apolipoprotein (apo) A-I and IL-6 concentrations were determined by immunoturbidimetry (Beckman-Coulter). Plasma viral load was measured with the Cobas® TaqMan HIV-1 assay (Roche, Basel, Switzerland) and CD4 T-cell count was determined by flow cytometry (Beckman-Coulter). The serum concentration of oxLDL was

measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden). The serum concentration of CRP was measured using a high-sensitivity method (Beckman-Coulter) [29]. The plasma concentration of MCP-1 was measured by ELISA (Human MCP-1 ELISA Development Kit; Peprotech, London, UK). Serum PON1 activity and concentration were analysed as previously reported [29,30].

CVD risk assessment

The 10-year CVD risk was assessed in all patients by applying the FRS. We categorized individuals on the basis of three levels of CVD risk: low (< 10%), moderate (10–20%) and high (> 20%).

Measurement of CIMT

CIMT measurements were obtained by ultrasonography as previously described using a GE Logiq 700MR system (General Electric, Milwaukee, WI, USA). We identified three segments of the carotid arteries on which to conduct the measurements, the common carotid artery (1 cm proximal to the bifurcation), the carotid bulb (in the bifurcation) and the internal carotid artery (1 cm distal to the bifurcation). Far wall CIMT images were obtained and digitalized for each patient [31]. The median value of the measurements obtained in the three segments was used in the statistical analyses. The presence of subclinical atherosclerosis was defined as a median CIMT > 0.8 mm or the presence of a plaque. A plaque was defined as a thickness > 1.5 mm or a focal structure that encroaches into the arterial lumen by at least 0.5 mm, or 50% of the surrounding CIMT value [32].

Statistical analysis

We used the χ^2 test or Fisher's exact test to evaluate the associations between categorical variables. The κ coefficient was used as a measure of the agreement between the presence of subclinical atherosclerosis and the CVD risk calculated by the FRS. Means for variables with a normal distribution were compared using analysis of variance (ANOVA) and the Kruskall–Wallis test was used for variables with nonnormal (skewed) distributions. When significant differences among CVD risk groups were found, pair-wise comparisons were performed between the groups representing the three levels of CVD risk. *Post hoc* analyses included the Bonferroni test. Logistic regression analysis was used to study the variables associated with the presence of subclinical atherosclerosis (as a binary variable) in the group of patients with low CVD risk as stratified by FRS (i.e. risk < 10%). Variables included in the multivariate analyses were age, gender, smoking status, systolic blood pressure (SBP), diastolic blood pressure (DBP), glucose, LDL cholesterol, HDL cholesterol, triglycerides, BMI, HIV-1 basal viral load, basal CD4 cell count, lipodystrophy, exposure time to nucleoside reverse transcriptase inhibitor (NRTI), nonnucleoside reverse transcriptase inhibitor (NNRTI) and protease inhibitor (PI) treatments, inflammatory markers, and oxidative markers. Statistical analyses were performed with the SPSS 17.0 statistical package (SPSS, Chicago, IL, USA). A significant difference was defined as two-tailed $P < 0.05$.

Results

Relationship between the presence of subclinical atherosclerosis and the FRS

We observed a low level of agreement between the stratification of CVD risk measured using the FRS and the presence of subclinical atherosclerosis measured using CIMT (Table 1). Of note is the finding that a high number of patients who did have subclinical atherosclerosis were classified as having a low CVD risk using the FRS score ($n = 66$; 56.4%).

Differences among groups stratified by CVD risk according to the FRS and the presence of subclinical atherosclerosis according to CIMT

Table 2 summarizes the data on the patients stratified into three groups according to the presence of atherosclerosis, but with a low or high CVD risk according to the FRS. The group of patients with a high CVD risk according to the FRS and with atherosclerosis were older ($P < 0.001$) and contained a greater proportion of males ($P < 0.001$) than the other groups of patients. With respect to HIV-related variables, we did not find any significant difference in immunological or virological status, or the time since diagnosis. There was a significantly higher proportion of patients with lipodystrophy among those with atherosclerosis and higher CVD risk ($P < 0.001$).

Table 1 Correlation between the presence of subclinical atherosclerosis assessed using carotid intima-media thickness (CIMT) and cardiovascular disease risk estimated using the Framingham risk score (FRS)

FRS (%)	No atherosclerosis based on CIMT [*n* (%)]	Atherosclerosis based on CIMT [*n* (%)]	*P*-value (Fisher's test)	κ (*P*-value)
< 10	51 (43.6)	66 (56.4)	< 0.001	0.229 (< 0.001)
10–20	1 (1.9)	27 (96.4)		
> 20	2 (20)	8 (80)		

Table 2 Characteristics of HIV-infected patients stratified according to the presence or absence of atherosclerosis as assessed using carotid intima-media thickness (CIMT) and the Framingham risk score (FRS)

Characteristic	No atherosclerosis FRS < 10%	Atherosclerosis FRS < 10%	Atherosclerosis FRS > 10%	*P*
Age (years)	35.9 (3.5)	39.0 (4.3)*	46.7 (7.8)†,‡	< 0.001
Gender (%)				0.073
Male	58	47	66	< 0.001
Female	42	53	34	
Hepatitis C virus coinfection (%)	18	29	11	0.160
Risk factors for HIV infection				0.809
Injecting drug use (%)	54	68	45	0.143
Male homosexual contact (%)	14	5	19	
Heterosexual contact (%)	32	27	36	
Months since HIV diagnosis	82 (53)	95 (49)	86 (52)	0.549
Basal CD4 cell count (cells/μL)	364.28 (313.05)	332.37 (276.49)	285.94 (207.56)	0.182
Viral load < 40 copies/mL (%)	60	69	74	0.565
Lipodystrophy (%)	12	35	43	< 0.001
AIDS-related disease (%)	28	33	37	0.070
Previous antiretroviral therapy (months)				
NRTIs	89 (65)	105 (67)	103 (58)	0.431
Protease inhibitors	28 (30)	29 (28)	32 (27)	0.385
NNRTIs	8 (10)	9 (11)	5 (9)	0.177
Cardiovascular disease risk factors				
Current smoker (%)	88	83	80	0.625
Fasting glucose (mmol/L)	5.18 (0.52)	5.25 (0.73)	6.02 (1.73)†,‡	< 0.001
BMI (kg/m^2)	19.65 (2.75)	18.79 (2.67)	19.76 (3.14)	0.138
SBP (mmHg)	114.05 (13.56)	117.25 (13.10)	134.28 (22.08)†,‡	< 0.001
DBP (mmHg)	74.5 (11.17)	75.81 (8.98)	85.21 (13.87)†,‡	< 0.001
CIMT (mm)	0.65 (0.07)	0.81 (0.14)*	0.93 (0.29)†,‡	< 0.001
Lipid profile				
Cholesterol (mmol/L)	4.7 (1.2)	4.7 (1.2)	5.7 (1.3)†,‡	< 0.001
LDL cholesterol (mmol/L)	2.6 (0.9)	2.64 (1.0)	3.4 (1.2)†,‡	0.003
HDL cholesterol (mmol/L)	1.4 (1.0)	1.2 (0.4)	1.0 (0.3)	0.113
Triglycerides (mmol/L)	1.74 (1.40–2.08)	1.92 (1.63–2.22)	3.38 (2.50–4.24)†,‡	< 0.001
Apolipoprotein A-I (mg/L)	1.41 (0.34)	1.36 (0.32)	1.33 (0.22)	0.621
Inflammation and oxidation markers				
IL-6 (mg/L)	0.68 (0.68)	0.69 (0.70)	0.86 (0.73)	0.467
CRP (mg/L)	4.27 (7.60)	3.90 (4.89)	4.09 (4.02)	0.899
MCP-1 (ng/L)	59.4 (24.3)	81.9 (57.4)*	67.2 (23.3)	0.006
PON1 activity (U/L)	307.2 (267.8–346.5)	340.6 (298.9–382.37)	347.6 (285.1–410.2)	0.784
PON1 concentration (mg/L)	158.3 (110.1–206.5)	164.2 (115.8–212.4)	174.7 (54.4–95.0)‡	0.006
oxLDL (U/L)	76.22 (28.43)	86.69 (31.34)	97.80 (29.22)†	0.013

Unless otherwise stated, results are presented as mean (standard deviation) for variables with parametric distributions, and as median (range) for variables with nonparametric distributions.
*$P < 0.05$ for comparison between the group 'No atherosclerosis and FRS < 10%' and the group 'Atherosclerosis and FRS < 10%'.
†$P < 0.05$ for comparison between the group 'No atherosclerosis and FRS < 10%' and the group 'Atherosclerosis and FRS > 10%'.
‡$P < 0.05$ for comparison between the group 'Atherosclerosis and FRS < 10%' and the group 'Atherosclerosis and FRS > 10%'.
BMI, body mass index; CIMT, carotid intima-media thickness; CRP, C-reactive protein; DBP, diastolic blood pressure; HDL, high-density lipoprotein; IL, interleukin; LDL, low-density lipoprotein; MCP, monocyte chemoattractant protein; NNRTI, nonnucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; oxLDL, oxidized LDL; PON, paraoxonase; SBP, systolic blood pressure.

With respect to the conventional CVD risk factors, we found statistically significant differences among the three groups in relation to blood pressure ($P < 0.001$), fasting glucose ($P < 0.001$), serum cholesterol ($P < 0.001$), LDL cholesterol ($P = 0.003$) and triglycerides ($P < 0.001$). We did not observe any significant differences with respect to HDL cholesterol and apoA-1 concentrations.

Of particular note in the present study was that plasma MCP-1 concentrations were significantly higher in patients with atherosclerosis, but with a low CVD risk, than in patients without atherosclerosis ($P = 0.006$). In addition, oxLDL and PON1 concentrations were significantly higher in patients with atherosclerosis and > 10% risk ($P = 0.013$ and $P = 0.006$, respectively) than in patients without atherosclerosis. Mean CIMT was significantly higher in patients with higher CVD risk [0.90 (0.29) mM *vs.* 0.74 (0.14) mM; $P < 0.001$].

Variables associated with the presence of subclinical atherosclerosis in the group of patients with < 10% FRS

In the logistic regression analysis, the variables that were significantly associated with the presence of subclinical atherosclerosis in patients with low estimated CVD risk were age [odds ratio (OR) 1.285; 95% confidence interval

(CI) 1.084–1.524; $P = 0.004$], BMI (OR 0.779; 95% CI 0.642–0.994; $P = 0.044$), oxLDL (OR 1.026; 95% CI 1.001–1.051; $P = 0.041$), and MCP-1 concentration (OR 1.027; 95% CI 1.004–1.050; $P = 0.020$).

Discussion

Viral suppression and immune reconstitution have become achievable goals in the treatment of HIV infection as a consequence of effective antiretroviral drugs becoming available under the various public health systems in developed countries [1]. However, CVD has increasingly been reported as a clinical complication of HIV infection [2]. The pathogenic factors associated with an increase in CVD risk in this relatively young population are mainly dyslipidaemia and insulin resistance related to antiretroviral treatment [3]. Chronic HIV infection together with the pro-oxidative and pro-inflammatory status of these individuals could also play an important role in the increase in CVD, as has been demonstrated previously [5].

Because there is still controversy regarding the application of these population-derived CVD risk scores to HIV-infected patients [12–15], we decided to assess the agreement of the FRS with the presence of subclinical atherosclerosis in a representative sample of this HIV-infected patient population. We found a good concordance between the estimated FRS and atherosclerosis (as measured by CIMT) in patients with FRS $\geq$ 10%. However, there was a high proportion of HIV-infected patients who had low CVD risk (as estimated by the FRS) but who already had evidence of atherosclerosis (as measured by CIMT). This observation is probably attributable to the impact of age in all these risk indices and to the fact that HIV-infected patients are usually young. This finding also supports the conclusion that different tools to address the clinical status of this patient population need to be developed. CIMT together with inflammatory and oxidative biomarkers may be useful measurements for a more precise CVD risk assessment in these patients. Carotid ultrasonography is a noninvasive diagnostic tool that provides a direct image of the arterial wall, and is strongly related to coronary atherosclerosis. Hence, CIMT is useful in making clinical decisions regarding implementation of therapy to prevent future adverse cardiovascular events. Also, the CIMT measurement enables the effect of treatments on atherosclerosis progression/regression to be evaluated in patient follow-up. Unfortunately, we have not measured CIMT in age- and gender-matched control subjects and we are therefore unable to present carotid thickness comparisons. However, a recent meta-analysis showed that CIMT in healthy populations is around 0.6–0.7 mm on average, similar to the values obtained in the present investigation in HIV-infected patients without atherosclerosis [16].

HIV-infected patients have a higher CVD risk, mainly because of lipid disturbances promoted by antiretroviral drugs, as well as the HIV infection itself. We found a higher rate of an abnormal fasting glucose, high blood pressure and lipodystrophy in the HIV-infected patients with atherosclerosis, reflecting insulin resistance associated with HIV infection and the antiretroviral drugs used [33,34]. Paradoxically, a low BMI was associated with greater CIMT. A low BMI in HIV-infected patients is often attributable to the wasting syndrome and immune system depletion. Hence, the elevated inflammatory and oxidative activities that characterize this situation could, at least in part, explain this correlation.

The results of the present study suggest that the chronic oxidative and inflammatory status related to HIV infection may explain the discrepancy we observed between the presence of subclinical atherosclerosis and the FRS. Indeed, plasma MCP-1 concentrations were significantly increased in patients with subclinical atherosclerosis and low CVD risk estimated by the FRS and, in the multivariate analysis, both serum oxLDL and MCP-1 concentrations were associated with the presence of atherosclerosis. This finding is of particular note as these biochemical parameters can be measured relatively easily in order to improve the ability to identify at-risk individuals. In addition, the relationship between these markers and vascular lesions suggests that anti-inflammatory and antioxidant treatments could assist in the management of CVD risk in these patients. However, a caveat is that the OR for the association between these parameters and the presence of atherosclerosis was relatively small. This is probably a reflection of atherosclerosis development in HIV-infected patients being a highly complex phenomenon, and other factors, as yet unknown, may be involved. This is illustrated by the observation that our patients with atherosclerosis and high FRS had an increased cholesterol but not MCP-1 concentration, in contrast to those with low FRS. Overall, most data suggest that traditional and nontraditional CVD risk factors may combine in a variety of ways to promote atherosclerosis. These factors warrant further investigation. Another point to be taken into account is the high prevalence of current smokers in our HIV-infected patients. This high prevalence of current smokers may be associated with a high incidence of injecting drug use, and may influence the mean circulating levels of oxidation and inflammation markers. Therefore, we cannot be certain that our conclusions can be generalized to other HIV-infected populations.

In conclusion, the assessment of CVD risk with FRS underestimates atherosclerosis in our HIV-infected patients. Apart from the classical CVD risk factors such

as dyslipidaemia, smoking habit, hypertension and diabetes, we propose that the measurement of CIMT, serum MCP-1 and serum oxLDL concentrations may be useful additional tools to evaluate more effectively the level of CVD risk in these patients.

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DISCUSSIÓ GENERAL

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La funció fisiològica de PON1 en diverses malalties cròniques que comporten un increment de l'estrès oxidatiu i l'aterosclerosi és una àrea d'investigació amb resultats que aporten coneixement sobre la funcionalitat de les partícules de colesterol HDL en diverses situacions fisiopatològiques.

La infecció crònica pel retrovirus VIH-1, ha esdevingut un paradigma de model fisiopatològic d'estat pro-oxidatiu (159-163) i pro-inflamatori amb complicacions metabòliques que cada cop precisen d'una atenció mèdica més complexa, com són diverses malalties associades i l'aterosclerosi (168).

La disminució de la mortalitat associada a la immunodepressió severa i les malalties definitòries de SIDA, ha promogut que cada cop els metges especialitzats en el seguiment i tractament d'aquests pacients hagin de tractar les complicacions clíniques degudes als efectes adversos dels fàrmacs antiretrovirals, a la coexistència d'altres malalties importants com les hepatopaties víriques, complicacions renals, òssies i una sèrie d'alteracions metabòliques relacionades amb alteracions del perfil lipídic i un increment de la resistència insulínica (216,217).

El possible efecte de la infecció pel virus del VIH sobre l'estat sèric de l'enzim PON1 està resumits a l'**estudi 1**. En aquest primer treball vam demostrar que l'estat de la infecció produïa alteracions en l'activitat i la concentració sèrica de l'enzim, tot i que no hi havia diferències genotípiques de PON1 respecte les variants al·lèliques estudiades entre els pacients infectats i la població control.

Vam objectivar que l'estat d'infecció reduïa significativament l'activitat sèrica de PON1 i augmentava la concentració de l'enzim, resultats que ens confirmaven la hipòtesi que l'alteració de l'equilibri redox dels pacients es podria veure reflexat en l'estat sèric de PON1. També vam observar en aquest primer estudi que les activitats de PON1 utilitzant el paraoxó com a substrat estaven determinades pels polimorfismes a les posicions $PON1_{192}$, $PON1_{55}$ i a la regió promotora $PON1_{-107}$ de la mateixa manera tant en la població control com en la població infectada i tal i com estava descrit prèviament a la literatura. D'aquesta forma es podien descartar, en principi, que les variacions de l'activitat sèrica de l'enzim observada al comparar les dues poblacions, estiguessin en relació a diferències genotípiques.

L'increment de la concentració sèrica de PON1 és una troballa que també s'ha descrit en altres patologies (74,75), hipotetitzant que pot tractar-se d'un increment de la síntesi per tal de contrarestar aquest ambient oxidatiu.

Els pacients amb replicació viral no suprimida presentaven una concentració sèrica de PON1 significativament més elevada i en l'anàlisi multivariant els nivells de β2-microglobulina, un marcador de replicació viral, també presentaven una forta associació positiva amb els nivells de PON1, suggerint que existeix una relació entre una major replicació viral i l'increment de concentració de PON1. També vam trobar que la coinfecció pel VHC produïa diferències en quant els nivells de l'activitat de PON1 tal i com s'ha descrit prèviament (74) tot i que també aquests pacients prentaven nivells més inferiors de LDL oxidada, probablement en relació amb uns nivells de LDL totals més baixos.

En el nostre treball no hem trobat que la presència de lipodistròfia produeixi alteracions del producte sèric de PON1, tot i que sí que presenten nivells més baixos d'HDL total i nivells més elevats de LDL oxidada. Aquests paràmetres reflexen les alteracions metabòliques associades a un major estrès oxidatiu i la presència de resistència insulínca present en la síndrome metabòlica i la lipodistròfia.

Per tant, en aquest estudi es demostra que la concentració i l'activitat de l'enzim PON1 estan influenciades per la infecció pel VIH i que aquestes alteracions es relacionen amb els nivells de HDL i apo-A1 i al mateix temps amb l'estat immunològic dels pacients.

En **l'estudi 2** vam profunditzar en l'anàlisi de les diferències dels genotíps de *PON1* respecte una població control més amplia, procedent de la mateixa àrea geogràfica. Vam realitzar l'estudi d'un major nombre de variants al·lèliques de *PON1* i vam comparar si existien diferències respecte la presència dels haplotips entre les dues poblacions. Aquests resultats van ser obtinguts gràcies a la col·laboració amb un grup de recerca especialitat en l'anàlisi d'estudis genètics, el Centre Nacional del Genotipat (CeGen).

Els resultats obtinguts es mostren en aquest treball, obtenint diferències estadísticament significatives per la presència de dos haplotips obtinguts a

partir dels SNPs de *PON1*. Aquests haplotips anomenats H10 (GTCCGTC) i H5 (GACCGTC) mostren també una associació estadística amb la capacitat per mantenir la supressió viral completa.

L'haplotip H10 (GTCCGTC) està associada a una major probabilitat de mantenir una supressio viral i també es troba en un percentatge significativament més elevat en la cohort de pacients infectats respecte el control. Per oferir una explicació a aquesta associació es podria especular que la presència de l'haplotip afavoriria un increment en la supervivència dels pacients. En canvi l'haplotip H5 (GACCGTC) es troba de forma significativament menys freqüent en els pacients infectats respecte la població general i la seva presència s'associa amb una major probabilitat per presentar fracàs en el manteniment de la càrrega viral, hipotetitzant que aquest genotip conferiria menys capacitat per controlar la replicació viral i fins i tot els pacients podrien ser més vulnerables en les primeres fases de la transmissió del virus.

Tot i aquests resultats seria necessari confirmar prèviament aquesta associació estadística en estudis poblacionals molt més amplis, i en diferents regions geogràfiques amb ètnies diferents. També seria interessant realitzar aquest estudis en cohorts de pacients resistents a l'exposició o fins i tot en poblacions amb alta barrera immunològica com els LTNP (Long Term Non Progressors).

Una altre haplotip diferent de PON1 el H7(AATTCCT) en canvi sembla tenir una funció important respecte el perfil lipídic, els nivells basals de CD4, la seva capacitat de reconstitució immune i la presència d'aterosclerosis subclínica.

En resum, en absència d'aquest haplotip els pacients presenten major proporció d'aterosclerosis subclínica i menors nivells de CD4 basals, d'HDL i lipoproteïna Apo-A1. La presència de l'haplotip es troba en la majoria de pacients que recuperen els nivells de CD4 després de l'inici de tractament antiretroviral.

L'associació entre aquest haplotip i els nivells de lipoproteïna apo A-1 i de colesterol HDL ens permeten hipotetitzar que els polimorfismes de *PON1* poden estar relacionats amb canvis conformacionals de la isoforma de PON1. Aquests canvis conformacionals conferirien una major estabilitat a la unió de PON1 i apo A-1 i un major nombre de partícules de colesterol HDL. De fet,

l'haplotip H7, conté l'al·lel Q a la posició $PON1_{192}$ que com hem explicat prèviament, és la isoforma de l'enzim que té millors propietats antioxidants.

Així doncs, sembla ser que la constitució genètica de PON1 confereix un estat oxidatiu més òptim en els pacients infectats pel VIH, repercutint en la modulació del perfil lipídic i els nivells de CD4. Com s'ha descrit en altres estudis (202,206), els nivells basals baixos de CD4 són un factor de risc per la presència d'aterosclerosis i esdeveniments cardiovasculars en els pacients infectats pel VIH. Possiblement menors nivells de CD4 basals són un reflexe o conseqüència d'un estat pro-oxidatiu que afavoreix, ell mateix, l'apoptosi i destrucció d'aquestes cèl·lules (163,164). Aquest mecanisme de depleció immunitària podria ser modulat per l'efecte antioxidant de PON1 i les partícules de colesterol HDL (49,156,161,178,205).

Per tant en els pacients infectats pel virus del VIH, l'estudi entre la interacció de l'enzim PON1 i HDL, juntament amb el coneixement de la seva predisposició genètica, podria ser d'utilitat per tal de considerar aquells pacients que es troben en major risc per presentar pitjor recuperació immunològica i més complicacions metabòliques. Aquest coneixement podria ser d'utilitat per tal d'optimitzar l'inici de tractament antiretroviral de forma més individualitzada, i fins i tot, plantejar estratègies terapèutiques alternatives per tal de potenciar l'activitat antioxidant de PON1 i la funció immunomoduladora de les HDL a través d'una homeòstasi de l'estrès oxidatiu dels pacients.

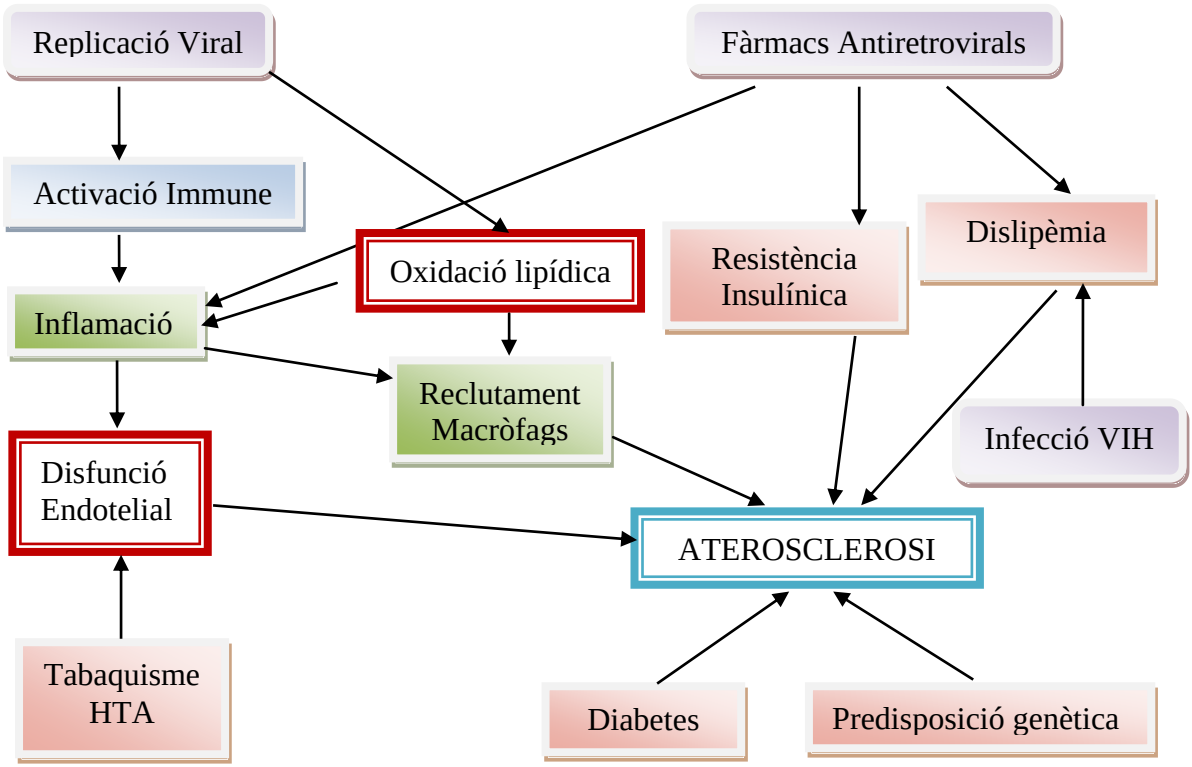
Una altra hipòtesi que ens vam plantejar en l'**estudi 3**, va ser l'aplicabilitat clínica de la determinació de l'activitat i la concentració sèrica de PON1 per avaluar el risc cardiovascular global dels pacients. Un estudi prospectiu sobre població general, no infectada, sí que va relacionar un descens de l'activitat sèrica de PON1 per tal de predir l'aparició d'esdeveniments cardiovasculars (144). Els estudis d'associació gènica de PON1 i malalties cardiovasculars tampoc han sigut concloents (83,142) arribant a la conclusió que potser és més important l'estudi de l'activitat i la concentració enzimàtica que els factors genètics de PON1, és a dir, el coneixement del "PON1 *status*" (78).

En aquest treball, també ens vam plantejar com a objectiu estudiar la concordança entre la presència d'aterosclerosi subclínica i la utilització

d'escales d'estimació de risc cardiovascular als 10 anys utilitzades habitualment en la població general no infectada. En aquestes escales no s'inclouen factors de risc "no clàssics" relacionats amb la inflamació i l'oxidació, que com hem esmenat, són importants en la patogènesi de l'aterosclerosi en aquesta població i poden donar més informació sobre el risc individual de presentar esdeveniments cardiovasculars.

En la **figura 16** es resumeixen els factors a partir dels quals es desenvolupa més aterosclerosi en els pacients infectats pel VIH.

Figura 16: Interaccions entre hoste, virus i tractament antiretroviral amb el risc de patir malaltia cardiovascular



En els resultats d'aquest treball vam observar que una gran proporció de pacients amb una estimació de risc cardiovascular baix, segons l'escala Framingham, en realitat ja presentaven aterosclerosi subclínica (56.4% dels pacients amb un FRS <10% als 10 anys). S'ha de tenir en compte que aquesta escala és de les més pessimistes en quant a l'estimació del risc cardiovascular

en comparació amb altres escales utilitzades en poblacions de risc més baix, com l'àrea mediterrània (REGICOR, SCORE).

En aquest estudi vam observar que a més dels factors de risc cardiovasculars clàssics associats a la presència d'aterosclerosi subclínica, els pacients amb aterosclerosi i risc baix presentaven nivells més elevats de la citoquina pro-inflamatòria MCP-1 i també nivells més elevats de LDL oxidada i concentració sèrica de PON1. No vam trobar diferències significatives entre els grups en funció de la presència d'aterosclerosi i risc cardiovascular en quan a nivells de Interleuquina-6 o proteïna C-reactiva. En aquell grup de pacients amb una estimació de risc baix i la presència d'aterosclerosi subclínica els nivells de MCP-1 i LDL oxidada a més de l'edat i l'índex de massa corporal eren els valors predictius de la presència de aterosclerosi subclínica. És a dir, a més dels factors de risc cardiovascular clàssics com tabac, HTA, perfil lipídic els pacients infectats pel VIH presenten un elevat risc de presentar malalties cardiovasculars donat a un increment de l'estat inflamatori i pro-oxidatiu. Per optimitzar l'avaluació del risc cardiovascular en pacients infectats pel VIH, s'haurien de valorar altres marcadors serològics d'aterosclerosi com els nivells de MCP-1 i LDL oxidada. Majors nivells plasmàtics de PON1 també semblen estar relacionats amb una major presència d'aterosclerosi però en l'anàlisi multivariant no sembla un predictor serològic de la presència d'aterosclerosi subclínica. Potser seria de major utilitat la utilització d'un substrat més específic per valorar la capacitat antiaterogènica de PON1 en aquesta població.

De fet, a l'estudi que s'indica a l'**annex 1**, s'observà que la utilització de diferents substrats (lactones o paraoxó), per determinar l'activitat de l'enzim PON1, sembla que reflexava diferents propietats antiinflamatòries i antioxidants que varien en funció de la presència de la infecció pel VIH.

Això podria ser explicat per diferents raons, com les interaccions entre el substrat enzimàtic, les proteïnes virals o els fàrmacs antiretrovirals. També és important tenir en consideració que la mida i les partícules d'HDL es veuen influenciades per la infecció crònica. Per tant, la utilització d'un substrat òptim per avaluar la funció fisiològica de PON1 podria donar informació més

específica sobre els efectes antioxidants i antiinflamatoris de l'enzim en aquesta situació clínica.

Una altra limitació del nostre estudi en quant a la utilitat de l'activitat paraoxonasa per valorar la capacitat funcional antioxidant de les HDL, és que només tenim una única determinació en un temps. L'estudi de la variabilitat de l'activitat de PON1 en diferents determinacions, en un mateix pacient al llarg del temps, podria correlacionar-se amb l'evolució clínica i immunològica dels pacients. En aquest sentit, l'estudi referit a l'**annex 2** demostra que els nivells d'activitat de PON1 realitzats de forma seriada en un temps donen una idea de la influència d'estats pro-inflamatoris i prooxidants en pacients afectes de lipodistròfia tractats amb diferents antidiabètics orals que disminueixen la resistència insulínica. L'estudi investiga sobre la influència negativa de l'estat postprandial en citoquines inflamatòries, els nivells d'activitat de PON1 i la resistència insulínica amb resultats que confirmen una menor resistència insulínica en aquells pacients amb nivells més elevats d'activitat paraoxonasa.

Per tant, l'estudi genotípic però també serològic de l'enzim PON1 pot ser d'utilitat en els pacients infectats pel VIH-1.

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Conclusions i Perspectives

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CONCLUSIONS

1. La concentració i l'activitat de PON1 estan influenciades per la infecció pel VIH. Els pacients amb replicació viral activa i nivells més elevats de β2-microglobulina presenten majors concentracions de PON1.

2. La presència de coinfecció per VHC s'associa a nivells d'activitat paraoxonasa inferiors.

3. Els haplotips de *PON1* estan associats a la infecció pel VIH, el metabolisme de les HDL, la presència d'aterosclerosi subclínica, així com a la seva recuperació immunològica.

4. La utilització de l'estimació de risc cardiovascular als 10 anys pel mètode de Framingham infradiagnostica els pacients VIH amb presència d'aterosclerosi subclínica.

5. La presència d'aterosclerosi subclínica està associada als factors de risc cardiovasculars clàssics i a un estat inflamatori i pro-oxidatiu.

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PERSPECTIVES

L'activitat de PON1 pot ser un biomarcador de la capacitat funcional del colesterol HDL amb efectes clínics sobre la regulació immunitària a través de la homeòstasi de l'estrès oxidatiu.

La valoració individual del risc cardiovascular en els pacients infectats pel VIH hauria d'incloure la utilització de marcadors d'aterosclerosis subclínica com la determinació del gruix íntima-mitja a caròtides i marcadors serològics d'inflamació i oxidació.

L'estudi de la funció antioxidant de PON1 en els pacients infectats pel VIH podria optimitzar-se amb la utilització d'un substrat enzimàtic més específic sense possibles interaccions amb els fàrmacs antiretrovirals i les partícules virals.

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ANNEXES

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ANNEX 1

PON1 paraoxonase and lactonase activity is influenced by HIV-infection. Enviat a Clin Chem and Lab Med

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Keywords: HIV infection; inflammation; oxidative stress; paraoxonase-1

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Abstract: Background: Paraoxonase-1 (PON1) is an antioxidant enzyme that attenuates the production of the monocyte chemoattractant protein-1 (MCP-1) in vitro. In the present study, we explored the relationships between the circulating levels of PON1 and MCP-1 in HIV-infected patients in relation to the multifunctional capabilities of PON1.
Methods: We measured selected variables in 220 HIV-infected patients and in a control group of 409 participants. Serum PON1 esterase and lactonase activities were analysed by measuring the rate of hydrolysis of paraoxon and 5-(thiobutyl)-butyrolactone, respectively. Serum PON1, oxidised LDL, and MCP-1 concentrations were determined by ELISA.
Results: There were significant relationships between PON1 activity and several indices of oxidation and inflammation in control subjects and in HIV-infected patients. However, our results indicated that such relationships may vary with disease status, or on the type of substrate used to measure PON1.
Conclusion: The present study is a cautionary tale highlighting that results of clinical studies on PON1 may vary depending on the methods used as well as the disease studied. We suggest the simultaneous employment of at least two different substrates. Also, the measurement of PON1 concentration is recommended in order to improve the reliability of the results obtained.

Type of article: Short communication

Paraoxonase-1: its measurement-dependent relationship with oxidative stress and monocyte chemoattractant protein-1 in HIV-infected patients

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Abstract

Background: Paraoxonase-1 (PON1) is an antioxidant enzyme that attenuates the production of the monocyte chemoattractant protein-1 (MCP-1) *in vitro*. In the present study, we explored the relationships between the circulating levels of PON1 and MCP-1 in HIV-infected patients in relation to the multifunctional capabilities of PON1.

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Conclusion: The present study is a cautionary tale highlighting that results of clinical studies on PON1 may vary depending on the methods used as well as the disease studied. We suggest the simultaneous employment of at least two different substrates. Also, the measurement of PON1 concentration is recommended in order to improve the reliability of the results obtained.

Keywords: HIV infection; inflammation; oxidative stress; paraoxonase-1

Paraoxonase-1 (PON1) is an enzyme with esterase and lactonase activities found in the circulation bound to high-density lipoproteins (HDL). Research into PON1 has increased exponentially over the past few years because many studies associate this enzyme with inflammation and cardiovascular disease. The physiological substrates of PON1 have not been completely delineated, but *in vitro* studies suggest that a key function is to degrade oxidised phospholipids in low-density lipoproteins (LDL) and HDL and, as such, has an antioxidant role (1). PON1 attenuates the production of the monocyte chemoattractant protein-1 (MCP-1) in cultured endothelial cells. MCP-1 is a pro-inflammatory chemokine involved in the initial steps in the formation of the atheromatous plaque (2). Previous studies from our group have shown that HIV-infected patients have decreased serum PON1 activities and increased plasma MCP-1 concentrations, and that the genetic polymorphisms of both molecules are associated with the presence of sub-clinical atherosclerosis (3-5). Although oxidation and inflammation are closely related processes, the association between PON1 and MCP-1 has not been completely characterised, as yet. This is probably due, at least in part, to the limitations of the current methods of measuring serum PON1 activity i.e. the current use of synthetic substrates limits the interpretation of the data since it is not clear to what extent these measured activities reflect the real endogenous physiological activity of the enzyme.

The present study was aimed at further investigating the relationships between the circulating levels of PON1 and MCP-1 in HIV-infected patients using two assay methods for the different enzyme activities (esterase and lactonase) and by measuring the PON1 protein concentration as well.

We studied 220 HIV-infected patients and 409 healthy volunteers. The exclusion criteria for the patients were age <18 years, or having an AIDS-related disease. The

clinical characteristics of these patients have been previously published (3). The study was approved by the Ethics Committee of our Hospital.

Serum PON1 esterase activity was measured as the hydrolysis of the substrate paraoxon (6), the lactonase activity was measured as the hydrolysis of the substrate 5-(thiobutyl)-butyrolactone (TBBL) (7), and the concentration of PON1 was determined by an in-house ELISA with a polyclonal antibody directed against a specific sequence of mature PON1(8). HDL-cholesterol and apolipoprotein (apo) A-I were analysed by standard methods (Beckman-Coulter, Fullerton, CA, USA), oxidised LDL (ox-LDL) by ELISA (Mercodia, Uppsala, Sweden), C-reactive protein (CRP) by a high sensitivity immunoturbidimetric method (Beckman-Coulter), and MCP-1 by ELISA (Prepotech, London, UK).

Normality of distributions was tested with the Kolmogorov-Smirnov test. We used Spearman's (ρ) test to identify correlations between variables, and curve estimation analyses when non-linear correlations were found. Statistical analyses were performed with the SPSS 17.0 statistical package. Results are presented as means (SD)

Results of the selected biochemical variables are shown in Table 1. There were significant decreases in serum PON1 activity and HDL-cholesterol and apolipoprotein A-I concentrations in HIV-infected patients with respect to the control group. Conversely, there were significant increases in plasma MCP-1 and serum CRP concentrations. oxLDL levels showed a slight trend to increase, but differences did not reach statistical significance. We observed a significant inverse relationship between serum PON1 esterase activity and oxLDL levels in the control subjects (linear; ρ = -0.125; p = 0.015), but not in the HIV-infected patients (Figure 1A). Conversely, we observed a significant curvilinear relationship between PON1 esterase activity and MCP-1 in HIV-infected patients (p = 0.023), but not in the control group (Figure 1B).

Esterase activity was also significantly correlated with HDL-cholesterol concentration in control subjects (linear; $\rho = 0.217$; $P < 0.001$) and in HIV-infected patients (curvilinear; $p = 0.004$). We did not find any statistically significant relationship between serum PON1 esterase activity and apo A-I or CRP in any of the studied groups.

Serum PON1 lactonase activity was significantly related to MCP-1 in the control subjects (linear; $\rho = 0.208$; $p = 0.002$) but not in HIV-infected patients (Figure 1C). Conversely, we observed a significant curvilinear relationship between lactonase activity and oxLDL ($p = 0.003$) in HIV-infected patients (Figure 1D), but not in the control group. We also found significant associations between serum PON1 lactonase activity and apo A-I (linear; $\rho = 0.281$; $P < 0.001$) and CRP (curvilinear; $p = 0.027$) in HIV-infected patients, but not in the control group.

The present study shows that the significant relationships between PON1 activity and several indices of oxidation and inflammation depend not only on the HIV infection but also on the substrate used to measure PON1. Methods using the non-physiological paraoxon as substrate have traditionally been used to measure PON1 activities, the levels of which have been proposed as indicators in the prediction of cardiovascular disease (1). Recently, the lactonase activity assay using TBBL has been developed and evaluated (7,9) and, although TBBL is also a synthetic substrate, it probably measures an activity that is closer to the physiological state. Experimental evidence suggests that, essentially, the lactonase assay measures PON1 that is tightly bound to HDL particles, while the esterase assay measures the tightly as well as the loosely bound enzyme (7). The differences between methods, therefore, could also be related to the observed changes in structure and composition of the HDL particles during HIV infection. These changes may be due to the chronic inflammation *per se*, or to secondary effects of the antiretroviral treatments (10). There is the possibility that

these changes affect PON1 activity and that this is further influenced by the type of substrate used to measure PON1. We acknowledge that the different relationships observed between PON1 activities and MCP-1 in HIV-infected patients may not be extrapolated, perhaps, to non-diseased control subjects. Nevertheless, the wide distribution of PON1 in different cell types and co-localisation with MCP-1 in most tissues (11) suggests a fundamental association between these two molecules in the regulation of oxidation and inflammation.

We conclude with a cautionary note to researchers conducting clinical studies on PON1 i.e. the present study highlights that the experimental results can vary depending on the methods of measurement used, and the disease studied. Until more specific methods using physiologically-akin substrates are developed for PON1 measurement, the simultaneous employment of at least two different substrates to measure PON1 activity, plus the measurement of PON1 concentration, would be the recommended evaluation in order to improve the reliability of the results obtained.

Conflict of interest statement

The authors declare that they do not have any conflict of interest

Acknowledgements

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Table 1 Results of the analytical measurements

Parameter	Control subjects	HIV-infected patients	p-value
PON1 esterase activity, U/L	410.7 (132.5)	336.8 (115.4)	< 0.001
PON1 lactonase activity, U/L	6.8 (3.0)	5.3 (1.6)	< 0.001
HDL-cholesterol, mmol/L	1.50 (0.01)	1.17 (0.03)	< 0.001
Apolipoprotein A-I, g/L	1.69 (0.03)	1.38 (0.31)	< 0.001
ox LDL, U/L	85.7 (80.1)	94.0 (90.8)	NS
MCP-1, ng/L	61.54 (1.97)	71.57 (2.80)	0.003
C-reactive protein, mg/L	0.75 (0.01)	4.13 (3.30)	< 0.001

Results are shown as means and SD (in parenthesis)

Figure legend

Figure 1 Relationships between serum PON1 esterase and lactonase activities and oxidised LDL and MCP-1 concentrations in the control group (open circles) and HIV-infected patients (closed circles). The lines within the graphs represent the linear or curvilinear regression lines of the distributions of measured values in the control group (dashed lines) and the HIV-infected patients (continuous lines). NS = not significant.

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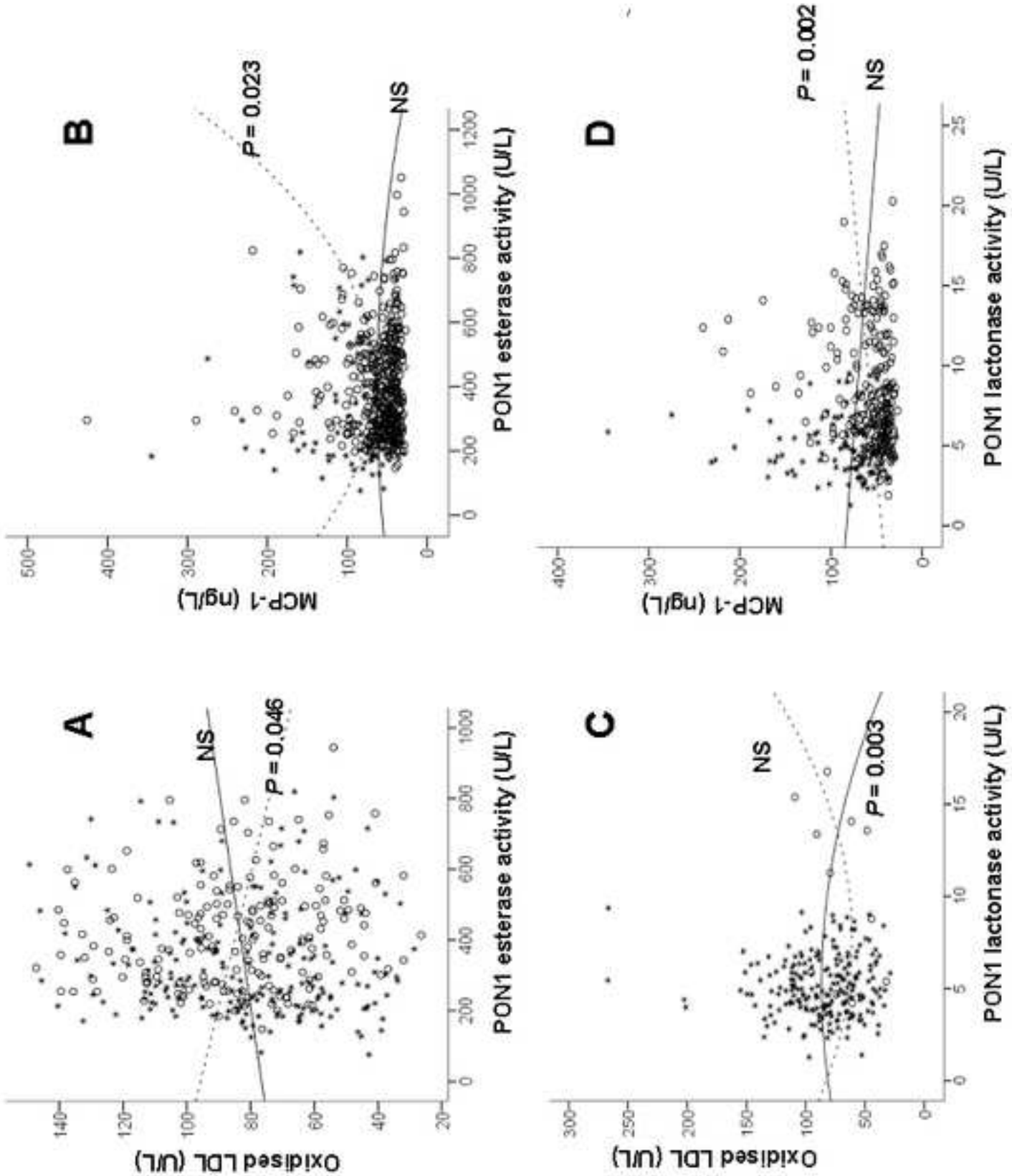

Figure 1
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ANNEX 2

Effects of rosiglitazone and metformin on postprandial paraoxonase-1 and monocyte chemoattractant protein-1 in human immunodeficiency virus-infected patients with lipodystrophy.Eur J Pharmacol. 2006 Aug 21;544(1-3):104-10. 2006 Jun 16.

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Effects of rosiglitazone and metformin on postprandial paraoxonase-1 and monocyte chemoattractant protein-1 in human immunodeficiency virus-infected patients with lipodystrophy

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Abstract

Highly active antiretroviral therapy in Human Immunodeficiency Virus (HIV) has been associated with lipodystrophy, insulin resistance and atherosclerosis. We investigated the effects of rosiglitazone or metformin on fasting and postprandial inflammatory and antioxidant variables in HIV-infected males with lipodystrophy.

Thirty-one patients were randomly assigned to receive either rosiglitazone (4 mg twice daily) or metformin (1 g twice daily) for 26 weeks. At baseline and after treatment, standardized 10-h oral fat loading tests were performed. Before treatment, inflammatory variables remained unchanged but there was a postprandial decrease in high density lipoprotein (HDL)-cholesterol and paraoxonase (PON1) activity. Rosiglitazone and metformin reduced homeostasis model assessment index (HOMA) similarly (−34% and −37%, respectively, $P<0.05$ for each). Both treatments increased fasting and postprandial PON1 activity and decreased postprandial monocyte chemoattractant protein 1 (MCP-1) concentrations. However, plasma C-reactive protein (CRP) and Interleukin-6 (IL-6) concentration did not change throughout the study.

To decrease insulin resistance results in a higher anti-oxidant and consequent lower pro-inflammatory action of HDL. This may confer protection against accelerated atherosclerosis in these patients.

Index words: HIV; Paraoxonase; Monocyte chemoattractant protein-1; Postprandial; Metformin; Rosiglitazone

1. Introduction

The use of highly active antiretroviral therapy (HAART) in Human Immunodeficiency Virus (HIV) has greatly reduced morbidity and mortality due to acquired immunodeficiency syndrome (AIDS) (Palella et al., 1998), but it is strongly associated with changes in fat distribution (lipodystrophy), dyslipidemia and insulin resistance, which increase the risk of atherosclerosis (Carr et al., 1998; Balasubramanyam et al., 2004; Hadigan et al., 2001; Friis-Moller et al., 2003). Atherosclerosis is generally accepted to be a low-grade inflammatory disease, initiated by the oxidation of lipoproteins in the subendothelial space, which induces chemotaxis and adhesion and transmigration of circulating monocytes into the arterial wall (Ross, 1999). Inflammation is mediated by cytokines. Particularly, the recruitment of monocytes to the subendothelial space is stimulated by monocyte chemoattractant protein 1 (MCP-1) (Gu et al., 1997). Other biomarkers of inflammation, C-reactive protein (CRP), has shown to be a direct toxic of endothelial cells (Libby and Ridker, 2004), and Interleukin-6 (IL-6) has been demonstrated to be higher in HIV-infected

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patients (Dolan et al., 2005), that represent predictors of cardiovascular events.

The enzyme paraoxonase 1 (PON1), located on High Density Lipoprotein (HDL), has potent antioxidant properties by hydrolyzing oxidized lipids formed on Low Density Lipoprotein (LDL) and HDL (Mackness et al., 1993). As such, it inhibits the oxidation of lipoproteins in the subendothelial space that, otherwise, could lead to the formation of foam cells. Supporting this concept, serum PON1 activity is low in patients with insulin resistance and atherosclerosis (Durrington et al., 2001; Mackness et al., 2000) and mice lacking serum PON1 activity are more susceptible to atherosclerosis (Shih et al., 1998). Furthermore, it has been demonstrated *in vitro* that PON1 attenuates the endothelial production of MCP-1 mediated by oxidized-LDL (Mackness et al., 2004). Therefore, PON1 has anti-inflammatory and anti-atherogenic properties and it is related to the action of MCP-1.

Since humans are non-fasting most part of the day, this period may be of particular importance in the pathogenesis of atherosclerosis. It is known that HIV-infected patients have delayed clearance of postprandial triglyceride-rich lipoproteins compared with healthy controls (Stein et al., 2005) and increased postprandial lipemia has been linked to accelerated atherosclerosis (Groot et al., 1991; Weintraub et al., 1996). The underlying mechanisms may involve increased generation of oxidative stress leading to endothelial dysfunction (van Oostrom et al., 2003; Ceriello et al., 2004). Metformin and rosiglitazone are used in clinical medicine to improve insulin resistance and glycemic control in patients with type 2 diabetes (Yki-Jarvinen, 2004; Hundal and Inzucchi, 2003). However, these agents may also have a role in treating patients with nondiabetic insulin-resistant conditions, such as HIV-lipodystrophy (Hadigan et al., 2000, 2002, 2004; Saint-Marc and Touraine, 1999; Carr et al., 2004; Sutinen et al., 2003). Presumptively, the treatment of the metabolic and inflammatory derangements may exert a protective effect from atherosclerosis in HIV-infected patients. We have studied the effects of a high-fat meal on inflammatory (MCP-1, CRP and IL-6) and antioxidant (PON1 activity and HDL-cholesterol) variables in HAART-treated HIV-infected patients with lipodystrophy, and investigated the effects of the insulin-sensitizing agents rosiglitazone and metformin on these variables.

2. Materials and methods

2.1. Study population

We included thirty-one males aged between 30 and 65 with a documented HIV infection and HIV-RNA values < 10,000 copies/ml, who were on HAART for at least 18 months with no changes in the treatment regimen during the 6 months prior to inclusion. We did not consider those with HIV-related symptoms (opportunistic infectious disease, malignancies or unexplained weight loss), renal, thyroid and/or liver diseases, diabetes mellitus and an alcohol intake > 3 U a day. The presence of HIV-lipodystrophy was defined as signs and symptoms of loss of subcutaneous fat (face, arms, legs and buttocks) with or without increased abdominal girth or development of a buffalo hump. The research project was primarily designed to study the effects of treatment on lipodystrophy course as well as endothelial function studies, the results of which have been already published (van Wijk et al., 2005).

2.2. Study design

At inclusion, a fasting blood sample was obtained and height, weight, blood pressure and waist and hip circumference were measured. A complete medical record was obtained and a thorough physical examination was performed. Participants underwent a standardized 10-h oral fat loading test, and were randomly assigned to receive either rosiglitazone (4 mg twice daily) or metformin (1000 mg twice daily) for 26 weeks. Patients visited the hospital after 2 and 4 months of treatment for safety evaluation. At the end of the period the second oral fat loading test was performed. The study protocol was approved by the local research ethics committees of the University Medical Center (Utrecht, the Netherlands) and the Hospital Universitari de Sant Joan (Reus, Spain).

2.3. Oral fat loading test

The subjects fasted overnight for at least 12 h and did not drink alcohol on the day before the test. After placing a cannula for venous blood sampling, subjects rested for 30 min before the fat load was administered. Fresh cream was used as the fat source. It was a 40% (weight/volume) fat emulsion with a poly-unsaturated/saturated fat ratio of 0.10, containing 0.001% (w/v) cholesterol and 3% (w/v) carbohydrates. The total energy content was 3700 kcal/l. Cream was ingested within 5 min at a dose of 50 g fat and 3.75 g glucose per m^2 body surface. The participants remained supine during each test and were only allowed to drink mineral water. Blood samples were collected into sodium EDTA-containing tubes for MCP-1, IL-6 and lipoprotein measurements, and most of the other biochemical analyses. Samples were collected into tubes with no anticoagulants added to measure PON1 activity. Samples were obtained before the fat load meal and at 2-h intervals up to 10-h postprandially.

Table 1
Selected baseline characteristics for each treatment group

	Metformin (N=16)	Rosiglitazone (N=15)	P value
Age, years	48.3 (1.9)	48.4 (1.8)	0.95
HIV diagnoses, years	8.4 (0.8)	12.2 (1.1)	0.01
Time under HAART*, years	7.6 (0.8)	9.06 (0.9)	0.23
ART prescribed, %			
Nucleoside analogues	100	100	
Protease inhibitors	61	68	0.59
Non nucleoside analogues	39	32	0.16
Cardiovascular risk factors (see also Table 2)			
Current smoker, %	22	26	0.71
Body Mass Index, kg/m^2	24.9 (0.4)	22.5 (0.6)	0.002
Systolic blood pressure, mm Hg	132 (3.45)	133 (3.73)	0.88
Diastolic blood pressure, mm Hg	81 (1.83)	82.2 (2.71)	0.77
HIV viral load, $\log_{10}$/ml	1.96 (0.14)	2.20 (0.22)	0.37
CD4 cell count, cells/mm^3	573 (58)	868 (87)	0.008

Unless otherwise indicated, values are mean (S.E.M.).
*HAART = highly-active antiretroviral therapy.

Table 2
Effects of rosiglitazone and metformin on fasting clinical and laboratory variables

	Overall (N=31)			Metformin (N=16)			Rosiglitazone (N=15)		
	Pre treatment	Post treatment	P value	Pre treatment	Post treatment	P value	Pre treatment	Post treatment	P value
Body Mass Index, kg/m^2	23.8 (0.4)	24.0 (0.3)	0.43	24.9 (0.4)	24.5 (0.4)	0.05	22.5 (0.6)	23.2 (0.5)	0.02
Waist to Hip Ratio, m	0.98 (0.01)	0.98 (0.01)	0.31	0.99 (0.01)	0.97 (0.01)	0.02	0.98 (0.01)	0.98 (0.01)	0.43
Glucose, mmol/l	5.51 (0.13)	5.15 (0.18)	0.003	5.64 (0.24)	5.30 (0.35)	0.10	5.37 (0.09)	5.0 (0.11)	0.004
Insulin, mU/l	7.90 (1.18)	5.64 (0.74)	0.005	8.06 (1.13)	5.51 (0.67)	0.03	7.72 (2.17)	5.78 (1.38)	0.08
HOMA index*	2.03 (0.34)	1.31 (0.19)	0.002	2.16 (0.43)	1.36 (0.26)	0.01	1.88 (0.54)	1.26 (0.28)	0.06
Total cholesterol, mmol/l	5.70 (0.16)	5.70 (0.23)	0.98	5.68 (0.24)	5.33 (0.28)	0.08	5.72 (0.24)	6.11 (0.35)	0.12
HDL cholesterol, mmol/l	1.12 (0.05)	1.10 (0.04)	0.67	1.03 (0.04)	1.10 (0.06)	0.17	1.22 (0.09)	1.11 (0.08)	0.06
LDL cholesterol, mmol/l	3.42 (0.15)	3.27 (0.18)	0.34	3.55 (0.22)	3.15 (0.25)	0.05	3.26 (0.20)	3.41 (0.28)	0.53
Triglycerides, mmol/l**	3.02 (0.53)	3.81 (0.47)	0.09	3.61 (0.94)	4.05 (0.77)	0.59	2.39 (0.43)	3.56 (0.56)	0.01
MCP-1, pg/ml	85.99 (5.33)	76.96 (3.20)	0.10	83.50 (6.71)	70.14 (2.71)	0.05	88.64 (8.56)	84.25 (5.45)	0.61
CRP, mg/l**	4.63 (0.93)	3.41 (0.71)	0.15	4.75 (1.58)	2.78 (1.20)	0.15	3.99 (1.20)	3.78 (0.99)	0.52
IL-6, pg/ml**	284.32 (56)	246.20 (51)	0.36	147.90 (23)	165.88 (66)	0.91	390.42 (105)	321.01 (84)	0.28
PON1, U/l	340.01 (30)	392.64 (33)	0.002	364.70 (48)	426.45 (52)	0.04	313.66 (35)	356.58 (42)	0.003

Values are expressed as mean (S.E.M.). *HOMA = Homeostasis Model Assessment. **These were log transformed for statistical purposes.

2.4. Laboratory measurements

Plasma glucose, total cholesterol and triglycerides were measured by standard methods. LDL was isolated by ultracentrifugation. HDL-cholesterol was analyzed using a homogeneous method (ITC Diagnostics, Barcelona, Spain). Insulin, plasma MCP-1 and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA) (Mercodia, Uppsala, Sweden and Peprotech, London, UK). The Homeostasis model assessment index (HOMA) was calculated as [fasting insulin (mU/l)×fasting glucose (mmol/l)]/22.5. The serum concentration of CRP was measured using a high sensitivity method (hs-CRP) (Quantex hs-CRP kit, Biokit, S.A., Barcelona, Spain) with a lower limit of detection of 0.10 mg/l. PON1 activity towards paraoxon was measured after the reaction of paraoxon hydrolysis into *p*-nitrophenol and diethylposphate, as described previously, Ferré et al. (2003).

2.5. Statistical analysis

Results are expressed as means (S.E.M.). Continuous variables were compared using analyses of variance (ANOVA) and the categorical variables using the Chi square test. During serial measurements, time effects were tested by repeated measures ANOVA. The effects of treatment were analyzed with paired *t*-tests, and in non-normal distributed variables log transformation was applied. To compare the differences between the effects of treatment an ANOVA of the mean change was applied. To evaluate the effect of treatment and during serial measurements, we took into account these variables that presented significant differences between groups in the baseline situation. Calculations were performed using SPSS 12.0 (SPSS Inc. Chicago, IL, USA). Statistical significance was taken at the 5% level. The size of the sample was calculated with β=0.10 and α=0.05 and based on a reduction of 20% in inflammatory variables. A multistep regression analyses was performed to study the variables that might have an influence on the response of MCP-1, PON1 activity, CRP and HDL cholesterol. We considered dependent variables ΔAUC MCP-1, ΔAUC PON1, ΔAUC CRP, ΔAUC IL-6 and ΔAUC HDL cholesterol, and independent variables those which had been correlated in a significant manner in a univariate analyses.

3. Results

3.1. General characteristics

Thirty-one HIV-infected patients were included in the study. The baseline characteristics of the study group are depicted in Table 1. Patients assigned to rosiglitazone were diagnosed of HIV infection significantly earlier and their CD4 cell count was higher than those patients in the group of metformin (Table 1).

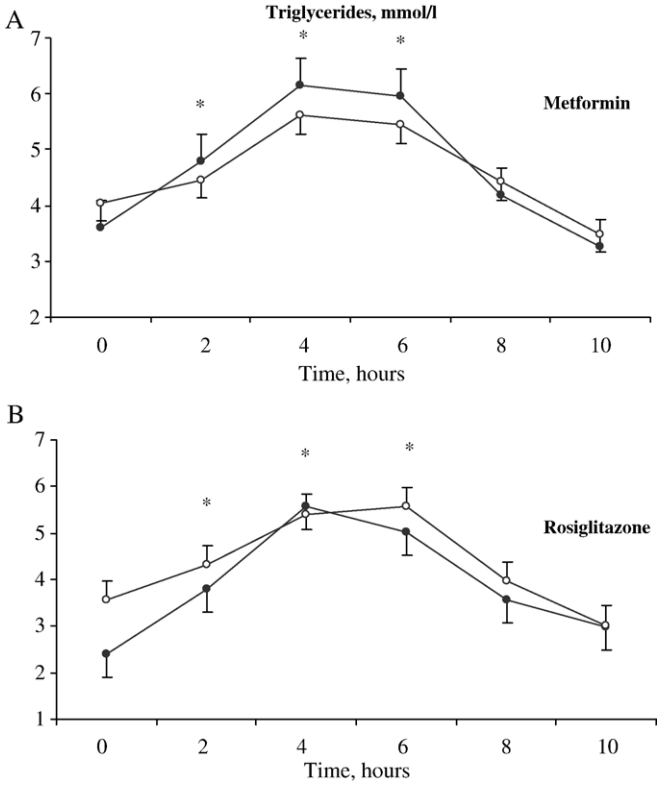

Fig. 1. Mean (S.E.M.) plasma triglyceride concentrations in HIV-infected patients before (●) and after treatment (○) in metformin (A) and rosiglitazone (B)-treated groups. There were no significant differences before and after treatment in the triglyceride response to fat overload. * Indicates P<0.01 comparing with t=0 when a paired t-test was applied.

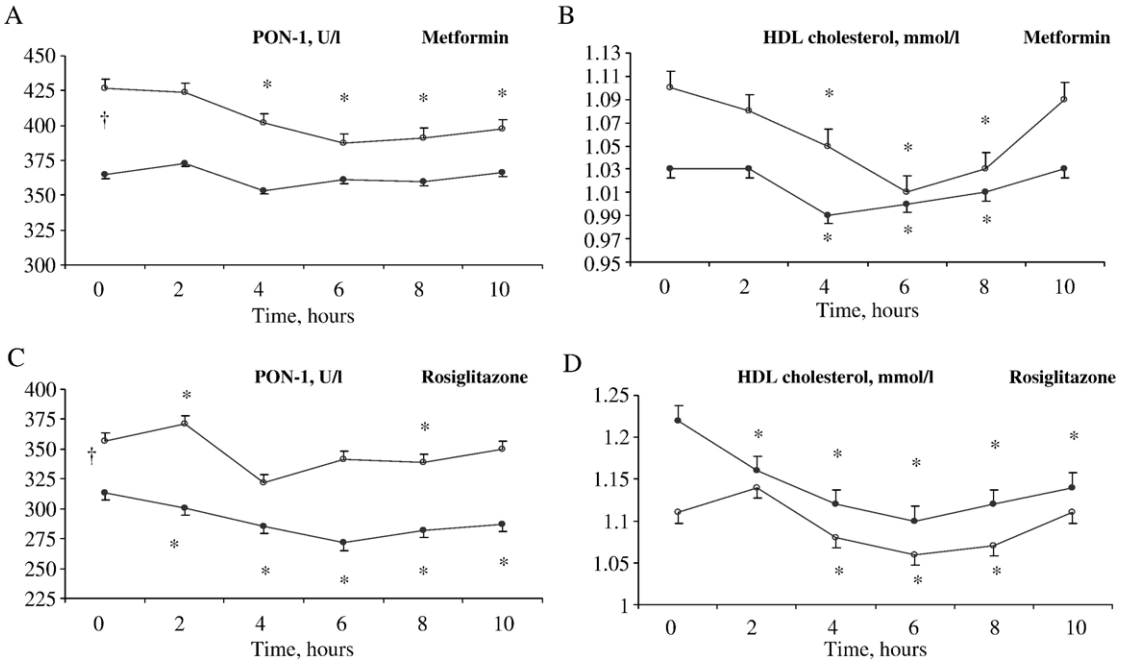

Fig. 2. Mean (S.E.M.) serum PON1 activity and HDL cholesterol in HIV-infected patients before (●) and after treatment (○) in metformin (panels A and B) and rosiglitazone-treated patients (Panels C and D). Although a significant ($P<0.05$) increase in fasting PON1 activity was observed in both groups (†), postprandial decreases were found significant (*$P<0.03$) when compared to $t=0$.

Further, patients in the group of metformin presented with a significant higher body mass index. All participants were on a nucleoside analogue agent plus a protease inhibitor and/or a non-nucleoside reverse transcriptase inhibitor. We did not find significant differences either in the baseline lipid profile or in the inflammatory (MCP-1, CRP) and anti-oxidant (PON1 activity and mass) variables, among patients assigned to receive rosiglitazone or metformin.

Both, rosiglitazone and metformin significantly decreased fasting insulin and HOMA index compared with baseline. Patients assigned to rosiglitazone, experienced a significant increase in the fasting triglyceride concentration (Table 2). Patients

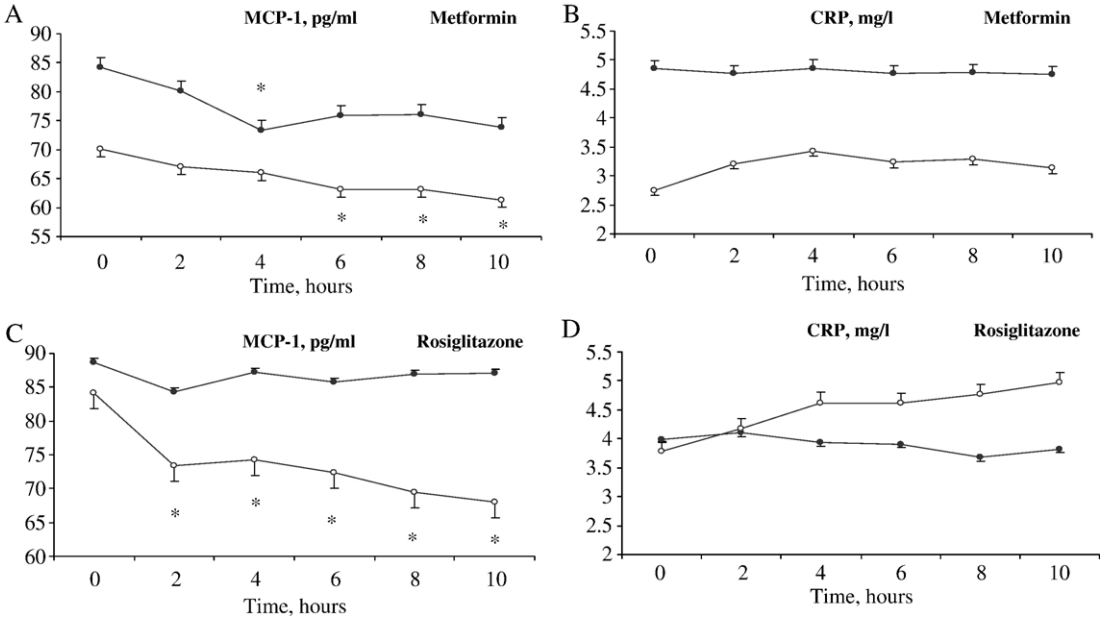

Fig. 3. Mean (S.E.M.) plasma MCP-1 and CRP concentrations in HIV-infected patients before (●) and after treatment (○) in metformin (panels A and B) and rosiglitazone-treated patients (panels C and D). We did not find significant differences among groups but there was a significant decrease in postprandial MCP-1 in the oral fat loading test performed after treatment with metformin and rosiglitazone *$P<0.01$, when compared to $t=0$.

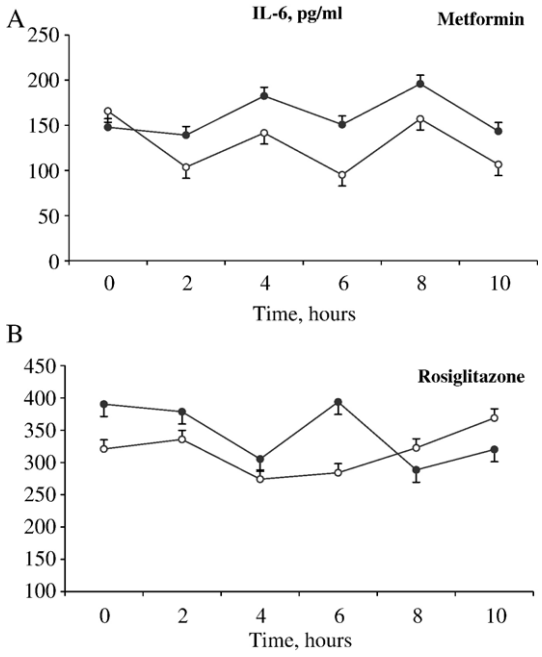

Fig. 4. Mean (S.E.M.) plasma IL-6 concentrations in HIV-infected patients before (●) and after treatment (○) in metformin (A) and rosiglitazone (B)-treated groups. There were no significant differences before and after treatment in the IL-6 response to fat overload.

assigned to metformin experienced a significant increase in fasting HDL cholesterol (0.06 mmol/l) when compared to those assigned to rosiglitazone (−0.10 mmol/l, $P=0.02$). Furthermore, in the patients on metformin, fasting total cholesterol showed a decrease (−0.35 mmol/l) that was significantly different when compared to those with rosiglitazone (+0.38 mmol/l, $P=0.02$ for treatment effect). We did not find significant differences between groups, before the randomization, in MCP-1, CRP, PON1 and IL-6.

3.2. Postprandial study

Plasma triglycerides increased significantly after fat ingestion, reaching maximum concentrations 4-h postprandially, and returning to baseline at the end of the test. Both, rosiglitazone and metformin did not change the AUC for triglycerides compared with the pre-treatment situation (Fig. 1). PON1 activity experienced a significant postprandial decrease throughout the postprandial test (Fig. 2). Both, metformin and rosiglitazone increased fasting PON1 activity (Table 2), but the postprandial course of PON1 showed a progressive and significant decrease in the postprandial period after both treatments. The course of HDL-cholesterol after the oral fat load was similar to that observed for PON1 activity, reaching a nadir 6 h postprandially (Fig. 2). Treatment with metformin or rosiglitazone did not significantly affect the response of HDL cholesterol to the oral fat load.

Before treatment, plasma MCP-1 decreased significantly in the group treated with metformin reaching a nadir at 4 h; however MCP-1 did not change after the oral fat load in rosiglitazone-treated participants (Fig. 3). After 26 weeks of treatment, however, fasting MCP-1 decreased with metformin, but not with rosiglitazone (Table 2). There was a progressive and significant decrease in plasma MCP-1 concentration in the postprandial period that was observed with both treatments. Metformin, but not rosiglitazone, tended to reduce fasting CRP levels, but there were no postprandial changes as a effect of treatment. Although patients assigned to receive rosiglitazone presented with higher concentration of plasma IL-6 before treatment, the difference was not statistically significant ($P=0.10$). Similarly, the postprandial response of IL-6 did not present significant variations when compared the effect of treatment and throughout the postprandial period (Fig. 4).

In a univariate analyses, we found a significant and positive correlation between the ΔAUC of MCP-1 and the increase in the total cholesterol and LDL cholesterol concentrations ($P=0.01$), but changes in insulin concentration or body mass index throughout the study did not correlate with changes in inflammatory variables, such as MCP-1 and CRP concentrations. Further, the ΔAUC of PON1 activity was correlated with HDL cholesterol ($P=0.02$). However, when a multistep, multivariate analyses was applied we did not find any significant association with any of the dependent variables in any of the treatment groups.

4. Discussion

As the survival of subjects with HIV increases, metabolic and vascular complications will become an increasingly important aspect in the management of these patients, specially those derived from accelerated atherosclerosis (Carr et al., 1998; Balasubramanyam et al., 2004; Hadigan et al., 2001; Friis-Moller et al., 2003). Since humans in Western societies are in a postprandial state most of the day, it is important to explore the postprandial period, which is thought to play an important role in the pathogenesis of atherosclerosis (Groot et al., 1991; Weintraub et al., 1996; van Oostrom et al., 2003; Ceriello et al., 2004). This effect will be presumably more intense in HIV-infected patients with lipodystrophy who show insulin resistance and increased postprandial lipaemia (Stein et al., 2005). These metabolic disturbances and the infection itself, represent a considerable degree of inflammation and oxidative stress to these patients. We determined the effects of two different insulin-sensitizing agents on fasting and postprandial inflammatory and antioxidant variables. We found that rosiglitazone and metformin both increased fasting PON1 activity, suggesting increased protection from oxidation. In addition, both treatments decreased the postprandial response of MCP-1, suggesting a reduction of the postprandial inflammatory response. These potentially beneficial effects may be the result of improved insulin sensitivity.

Both treatments significantly increased fasting PON1 activity, despite their different modes of action. Metformin mainly acts by decreasing hepatic glucose output, while the molecular mechanisms underlying the improved insulin sensitivity remain controversial (Hundal and Inzucchi, 2003). Rosiglitazone improves peripheral insulin sensitivity through transcriptional mechanisms (Yki-Jarvinen, 2004). It is likely that increased insulin sensitivity, which occurred similarly in both groups, may partially explain the observed effects on PON1 activity (Yamada et al., 2001; Mackness et al., 1998; Senti et al., 2003), however, these variables

were not significantly correlated in a linear regression model. Moreover, insulin supplementation in apolipoprotein E-deficient mice reduced the atherosclerotic lesion size by 22–37%, and showed a concomitant 30% increase in PON1 activity and an 18% reduction in lipid peroxide levels (Shamir et al., 2003). The postprandial response of PON1 was consistent with this concept and PON1 activity was higher at all time points which is probably due to a significant increase in fasting serum PON1 activity. Whether the increment in PON1 activity produced by rosiglitazone or metformin in our patients translates into clinical benefit with a reduced cardiovascular risk remains to be shown. We also expected changes in inflammatory variables. Serum CRP is a reliable and easily accessible marker of inflammation and is a strong predictor of cardiovascular events (Libby and Ridker, 2004). We found that the postprandial serum CRP concentrations did not change. Previous studies in type 2 diabetic patients treated with rosiglitazone have shown marked reductions in serum CRP concentrations (Haffner et al., 2002; Mohanty et al., 2004) and the postprandial concentration of IL-6 experimented a significant increase in type 2 diabetic patients (Ceriello et al., 2005). However, we should take into account that our HIV-infected patients are under an inflammatory stimulus which is probably unaffected by insulin-sensitizing agents. Also, the population studied was receiving HAART, which may have direct effects on inflammation. Metformin tended to reduce serum CRP concentration but did not reach statistical significance. However, we should take into account that larger trials assessing these variables should be of great value, since we are aware of the limitation of our study due to, at least in part, to sample size. The lack of a placebo control group is a limitation of our study; it should be of great value to perform the same design in non-HIV-infected participants with the concomitant presence of classic cardiovascular risk factors, such as patients with high blood pressure, diabetes or overweight. This comparison could give us interesting data in recognizing HIV infection as a pro-atherogenic status.

MCP-1 plays a crucial role in initiating atherosclerosis by recruiting inflammatory cells to the subendothelial space (Gu et al., 1997). Although the response of MCP-1 to a high-fat challenge has not been studied before, *in vitro* studies have suggested that chylomicrons induce a higher expression of MCP-1 (Domoto et al., 2003). At baseline, MCP-1 levels did not change postprandially. However, both metformin and rosiglitazone modified the postprandial response of MCP-1 by reducing the postprandial plasma concentrations of MCP-1. This may be a beneficial effect, because MCP-1 has been identified as a crucial mediator of atherosclerosis, and its plasma concentration has been positively correlated with subclinical atherosclerosis (Deo et al., 2004). We have also shown that HIV-infected patients with the MCP-1−2518G allele, which increases the expression of the MCP-1 gene, have a 5-fold increased risk of atherosclerosis (Alonso-Villaverde et al., 2004). Such a postprandial decrease in MCP-1 observed after treatment might be the direct result of improved insulin sensitivity, but the changes in body weight may be an additional factor to be considered (Christiansen et al., 2005).

In summary, a high-fat meal has only minor effects on inflammatory and antioxidant variables in HIV-infected patients with lipodystrophy, but treatment with metformin or rosiglitazone increased fasting and postprandial protection from oxidation and reduced the postprandial pro-inflammatory response by reducing plasma MCP-1 concentrations.

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