INFLAMACIÓN Y ESTRÉS OXIDATIVO EN EL SÍNDROME DE ISQUEMIA CRÓNICA DE MIEMBROS INFERIORES

Raúl Garcia Vidal

Dipòsit Legal: T. 191-2013

Raúl García Vidal

INFLAMACIÓN Y ESTRÉS OXIDATIVO EN EL SÍNDROME DE ISQUEMIA CRÓNICA DE MIEMBROS INFERIORES

TESIS DOCTORAL

dirigida por

Dr. Vicente Martín Paredero

Dr. Jorge Joven Maried

Dra. Anna Rull Aixa

Departament de Medicina i Cirurgia

UNIVERSITAT ROVIRA I VIRGILI

Tarragona 2012

UNIVERSITAT ROVIRA I VIRGILI

FACULTAT DE MEDICINA I CIÈNCIES DE LA SALUT
DEPARTAMENT DE MEDICINA I CIRURGIA

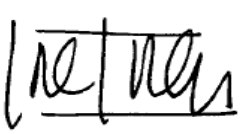
Carrer Sant Llorenç, 21
43201 Reus
Tel. 977 759 306
Fax. 977 759352

FEM CONSTAR que aquest treball, titulat "**INFLAMACIÓN Y ESTRÉS OXIDATIVO EN EL SÍNDROME DE ISQUEMIA CRÓNICA DE MIEMBROS INFERIORES**", que presenta **Raúl García Vidal**, ha estat realitzat sota la nostra direcció i que el doctorand acompleix els requeriments per poder optar al títol de Doctor.

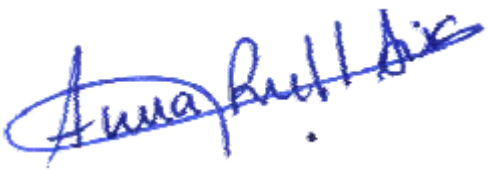
Reus, 27 de Febrer de 2012

Dr. Vicente Martín Paredero
Director de la tesi

Dr. Jorge Joven Maried
Codirector



Dra. Anna Rull Aixa
Codirectora



"Si sólo puede ir mejor y está cerca el momento
…espera que sople el viento a favor"

E.Bunbury

UNIVERSITAT ROVIRA I VIRGILI
INFLAMACIÓN Y ESTRÉS OXIDATIVO EN EL SÍNDROME DE ISQUEMIA CRÓNICA DE MIEMBROS INFERIORES
Raúl Garcia Vidal
Dipòsit Legal: T. 191-2013

AGRADECIMIENTOS:

- A TODAS las enfermeras del Servicio de Cirugía Vascular del H.U. Joan XXIII por su ayuda desinteresada.

- Al Dr. Vicente Martín Paredero, por animarme a participar en este proyecto.

- A la gente del CRB de Reus, especialmente al Dr. Joven y a la Dra. Anna Rull.

- A David, Laura, Mario y Yago.

- A Susana, Carla y Silvia, por hacer mis días tan entretenidos.

- A mamá...

...y a papá.

PRESENTACIÓN Y JUSTIFICACIÓN

UNIVERSITAT ROVIRA I VIRGILI
INFLAMACIÓN Y ESTRÉS OXIDATIVO EN EL SÍNDROME DE ISQUEMIA CRÓNICA DE MIEMBROS INFERIORES
Raúl Garcia Vidal
Dipòsit Legal: T. 191-2013

Las enfermedades cardiovasculares son la primera causa de muerte en España. Suponen más de un tercio de los fallecimientos registrados en nuestro país y algo más de cinco millones de estancias hospitalarias, con unas cifras totales de cerca de 20 millones de muertes anuales en todo el mundo. El incremento de los factores de riesgo, como la obesidad y la diabetes, alertan sobre la posible pandemia de enfermedad arterial periférica (EAP) en los próximos años, todo ello a pesar de una mayor concienciación de la importancia del estilo de vida.

La supervivencia en los pacientes con EAP es pobre. La mortalidad a los 5, 10 y 15 años en los pacientes con claudicación intermitente se encuentra alrededor del 30%, 50% y 70%, respectivamente. La supervivencia de los pacientes con isquemia crítica de miembros inferiores es peor. La mortalidad de los pacientes con dolor en reposo alcanza el 70% a los 5 años y el 85% a los 10 años.

Alrededor del 80% de los pacientes con EAP fallecen por una complicación vascular: más del 60% por una enfermedad coronaria y el 10% por accidentes cerebrovasculares. Los datos indican que la mortalidad se relaciona directamente con el grado de isquemia crónica de las extremidades inferiores determinada de forma objetiva en el momento de la presentación.

La evolución de los pacientes con claudicación es cada vez más maligna en cuanto a accidentes cardiovasculares graves, mortales y no mortales. Alrededor del 5% de los pacientes necesitará revascularización en un periodo de cinco años por claudicación grave o por evolución a isquemia crítica. Gracias a estos procedimientos, los índices de amputación se han reducido considerablemente, pero aún así, sigue siendo una causa de gran invalidez con costes sociales enormes.

Todos estos datos de morbimortalidad nos indican que estamos diagnosticando y tratando fases avanzadas de la enfermedad arteriosclerótica, pero existe un vacío importante en el diagnóstico en las fases iniciales, en las que sería posible practicar una prevención más efectiva

Existen diversos factores que dañan el endotelio y que generan una respuesta inflamatoria y fibroproliferativa que es excesiva. Es en este exceso donde se genera la enfermedad. El estrés oxidativo y la inflamación juegan un papel fundamental en la arteriosclerosis.

En el primer estudio exploramos si la relación inversa entre mecanismos de defensa endotelial y el estrés oxidativo, y la consiguiente respuesta inflamatoria se puede detectar en sangre. Hallamos que los resultados obtenidos en las concentraciones de MCP-1 y PON1, así como en las actividades lactonasa y paraoxonasa de PON1, prueban nuestra hipótesis y por tanto MCP-1 y PON1 constituyen nuevos marcadores del estado de enfermedad que se podrían utilizar como biomarcadores, especialmente

el ratio entre la concentración de MCP-1 y PON1, que claramente distingue entre los pacientes y los controles.

En el segundo estudio, hallamos que la concentración de PON3 está incrementada en pacientes con coronariopatía y con EAP, dos enfermedades asociadas por la arteriosclerosis pero con muchas diferencias en las alteraciones bioquímicas. Observamos que mientras el incremento de la concentración plasmática de PON3 se encuentra relacionada a la resistencia a la insulina en la EAP, en el grupo de pacientes con coronariopatías este aumento se correlaciona con un aumento en la concentración en sangre de marcadores de inflamación.

Dipòsit Legal: T. 191-2013

Dipòsit Legal: T. 191-2013

ÍNDICE

Dipòsit Legal: T. 191-2013

Dipòsit Legal: T. 191-2013

I *INTRODUCCIÓN*

Dipòsit Legal: T. 191-2013

CAPÍTULO 1

EPIDEMIOLOGÍA DE LA ARTERIOPATÍA PERIFÉRICA

Incidencia y prevalencia de la enfermedad arterial periférica asintomática.

La prevalencia total de la enfermedad arterial periférica (EAP) según análisis objetivos ha sido evaluada en varios estudios epidemiológicos y oscila entre el 3% y el 10%, un porcentaje que aumenta hasta el 15% y el 20% en las personas mayores de 70 años[1-6].

La prevalencia de la EAP asintomática en las extremidades inferiores sólo puede calcularse en la población general con medidas incruentas. La prueba más habitual consiste en la determinación del índice de presión sistólica entre el tobillo y el brazo (índice tobillo-brazo, ITB). En los individuos sintomáticos, un ITB ≤ 0,90 posee una sensibilidad del 95% para detectar una arteriopatía periférica confirmada por arteriografía y una especificidad cercana al 100% para identificar a los individuos sanos. Con este criterio varios estudios han analizado pacientes con arteriopatía periférica asintomática y sintomática en una misma población. La relación entre los dos tipos de pacientes resulta independiente de la edad y normalmente oscila entre 1:3 y 1:4 [7-8].

Incidencia y prevalencia de la enfermedad arterial periférica sintomática.

La claudicación intermitente (CI) se diagnostica normalmente conforme a los antecedentes de dolor muscular en la pierna que aparece durante el esfuerzo y que se alivia con un breve reposo. Se han elaborado varios cuestionarios para uso epidemiológico. Por lo que respecta a los métodos para identificar la CI en la población, debemos recordar que si bien es el principal síntoma de la EAP, su evaluación no siempre predice la presencia o ausencia de EAP.

Es posible que un paciente con EAP grave no presente dicho síntoma debido a otras afecciones que limitan el esfuerzo o bien porque muestra unos hábitos sedentarios. Por el contrario, algunos pacientes que parecen tener CI pueden no sufrir EAP. De

forma similar, los pacientes con una EAP muy leve pueden manifestar síntomas de CI sólo cuando realizan una actividad física intensa.

La incidencia anual de CI es más difícil de cuantificar y probablemente es menos importante que su prevalencia (a diferencia de lo que sucede con el mucho menor número de pacientes afectados por una isquemia crítica de la extremidad (ICE)). La prevalencia de CI parece aumentar desde alrededor de un 3% en los pacientes de cuarenta años hasta el 6% en los pacientes sexagenarios. En varios estudios poblacionales de gran tamaño se ha analizado la prevalencia de la CI. Un hallazgo sorprendente en los estudios de cribado poblacional es que entre el 10% y el 50% de los pacientes con CI nunca han consultado con un médico sobre sus síntomas[1-10].

EVOLUCIÓN DE LAS EXTREMIDADES

Pacientes asintomáticos

Las evidencias indican que la progresión de la EAP subyacente es idéntica tanto si el sujeto tiene síntomas como si no tiene síntomas en las piernas. Nada parece indicar que el riesgo de deterioro local con progresión a ICE depende de la presencia o ausencia de síntomas de claudicación intermitente.

El desarrollo de los síntomas depende principalmente del nivel de actividad del sujeto. Esta es una de las razones por las que la presentación inicial de algunos pacientes es con una ICE en ausencia de CI anterior. Por ejemplo, un paciente que tiene una disminución del ITB inmediatamente por encima del nivel de dolor isquémico en reposo pero que lleva una vida demasiado sedentaria como para presentar claudicación, puede desarrollar ICE debido a las heridas que no cicatrizan debido a este nivel de perfusión. Es importante detectar este subgrupo de pacientes en un momento en el que el cuidado protector de los pies y el control de los factores de riesgo tienen su máximo potencial de mejorar la evolución. El deterioro funcional en dos años está relacionado con el ITB basal y con la naturaleza de los síntomas de presentación en la extremidad [9].

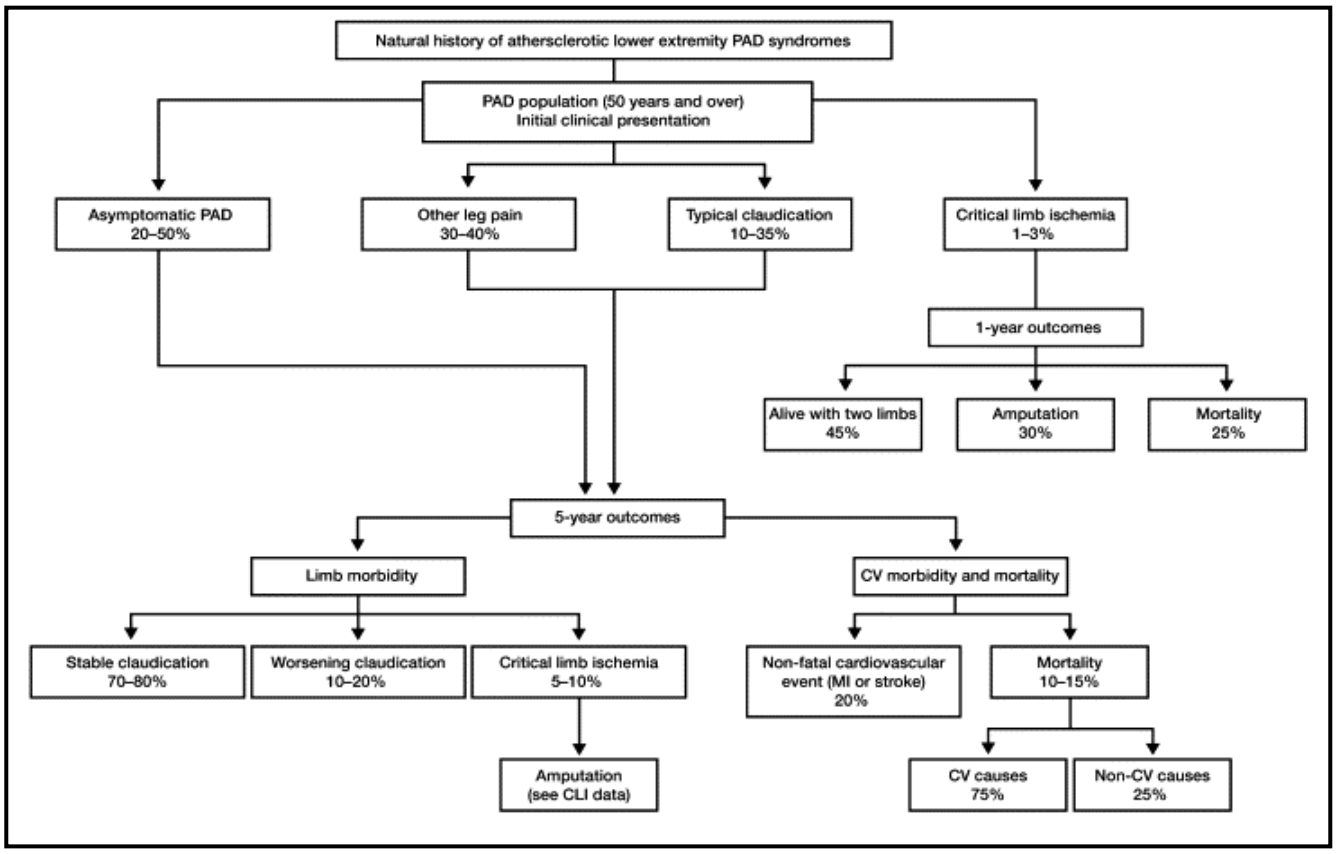


Fig.1. Evolución de los claudicantes a los 5 años. TASC II. Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC II). Eur J Vasc Endovasc Surg 2007;33,S1-S75.

Pacientes con claudicación intermitente

Aunque la EAP es una enfermedad progresiva en términos patológicos, su evolución clínica en lo que se refiere a la pierna es sorprendentemente estable en la mayoría de los casos. Sin embargo, los pacientes con EAP sintomática continúan teniendo una discapacidad funcional significativa. Los estudios poblacionales de gran tamaño aportan los datos más fiables. Todas las evidencias recopiladas en los últimos 40 años no han podido alterar sustancialmente la impresión de que aproximadamente sólo una cuarta parte de los pacientes con CI tendrá alguna vez un deterioro significativo. Esta estabilización sintomática puede deberse al desarrollo de colaterales, a la adaptación metabólica del músculo isquémico o a que el paciente altere su marcha a favor de los grupos musculares no isquémicos. El estadio clínico se deteriora en el 25% restante de pacientes con CI, con mayor frecuencia en el primer año después del diagnóstico (7%-9%) frente al 2% al 3% por cada año posteriormente. Esta estabilidad clínica es relevante para la percepción que tiene el paciente de la gravedad de su claudicación. Cuando estos pacientes son objeto de una evaluación exhaustiva de su situación

funcional real, la distancia caminada medida va deteriorándose progresivamente a lo largo del tiempo [9].

En las revisiones más recientes también se resalta que la amputación mayor es un resultado relativamente raro de la claudicación, necesitando una amputación mayor sólo el 1% al 3,3% de los pacientes con CI en un periodo de 5 años [10-11].

Es difícil predecir el riesgo de deterioro de una claudicación reciente. Los distintos factores de riesgo mencionados podrían contribuir a la progresión de la arteriopatía periférica. El cambio del ITB es, posiblemente, el mejor factor pronóstico individual porque si el ITB de un paciente se deteriora con rapidez, lo más probable es que continúe haciéndolo en ausencia de un tratamiento satisfactorio. Se ha demostrado que el mejor factor pronóstico del deterioro de la EAP en los pacientes con CI es un ITB < 0,50 con un cociente de riesgos > 2 frente a los pacientes que tienen un ITB > 0,50. En los estudios también se ha indicado que aquellos pacientes con CI en el estrato más bajo de presión en el tobillo (es decir, 40-60 mm Hg), el riesgo de progresión a isquemia importante o pérdida de la extremidad es del 8,5% por año.

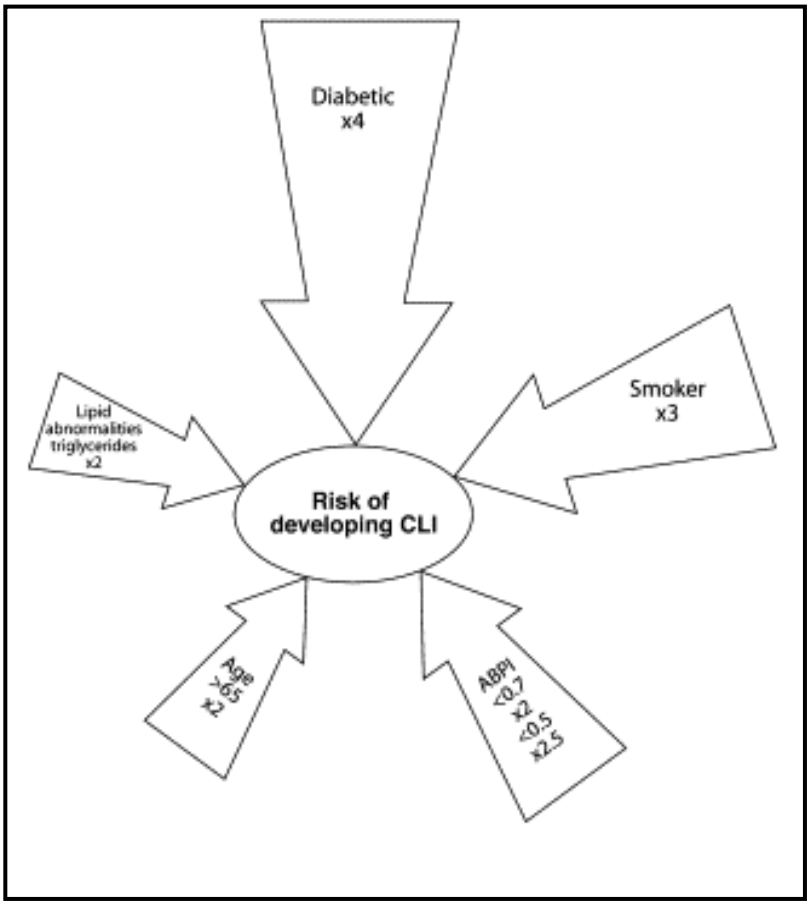


Fig. 2. Magnitud de los factores de riesgo en el desarrollo de la isquemia crítica de las extremidades en pacientes con enfermedad arterial periférica. TASC II. Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC II). Eur J Vasc Endovasc Surg 2007;33,S1-S75.

Pacientes con isquemia crítica de las extremidades

Los únicos estudios poblacionales prospectivos de gran tamaño fiables sobre la incidencia de la ICE ofrecieron una cifra de 220 casos nuevos cada año por millón de habitantes. Sin embargo, existen indicios indirectos obtenidos en estudios que analizaban la progresión de la CI, encuestas poblacionales sobre la prevalencia y suposiciones basadas en las tasas de amputación mayor. Sorprendentemente, la incidencia calculada utilizando estos diferentes métodos es muy similar y varía aproximadamente entre 500 y 1000 casos nuevos de ICE cada año en una población europea o norteamericana de un millón de sujetos [12].

En varios estudios se ha podido realizar un análisis de los factores de riesgo que parecen estar relacionados con el desarrollo de ICE. Dichos factores parecen ser independientes y, por tanto, probablemente sean aditivos [10-14].

Ya no es posible describir la evolución natural de los pacientes con ICE porque la mayoría de ellos recibe en la actualidad alguna forma de tratamiento activo. El tratamiento depende mucho del centro al que se derive el paciente. Las encuestas de gran tamaño indican que aproximadamente la mitad de los pacientes con ICE se someterá a algún tipo de revascularización, aunque en algunos centros intervencionistas particularmente activos se ha descrito un intento de reconstrucción hasta en el 90% de estos pacientes [13].

Existen algunos datos de buena calidad procedentes de estudios multicéntricos controlados estrictamente sobre el tratamiento farmacológico de la ICE. Estos estudios se refieren únicamente a un subgrupo de pacientes en los que no es posible la reconstrucción o en los que los intentos de reconstrucción han fracasado. Los resultados de este subgrupo ofrecen una pésima perspectiva en cuanto a que aproximadamente el 40% de los casos perderá la pierna antes de seis meses y hasta el 20% fallecerá [13].

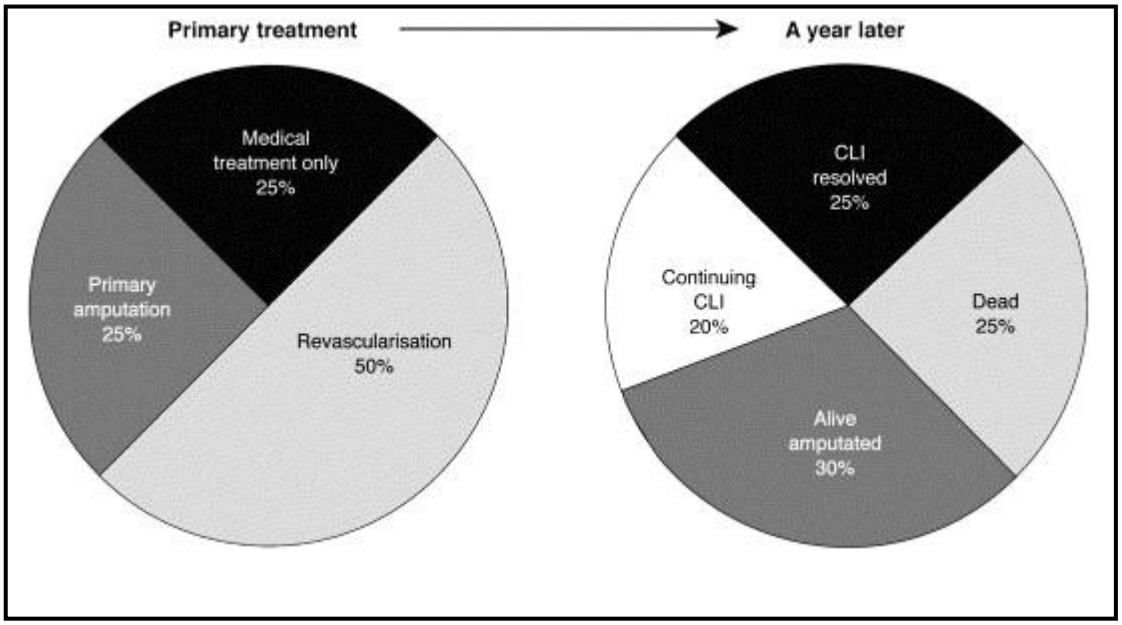


Fig. 3. Evolución de los pacientes con isquemia crónica crítica de la pierna. TASC II. Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC II). Eur J Vasc Endovasc Surg 2007;33,S1-S75.

Amputación

Se mantiene la controversia, a menudo alimentada por datos de revisiones retrospectivas no verificados de poblaciones de gran tamaño y en continua evolución, sobre si se produce una reducción significativa de las amputaciones como consecuencia de que se realizan más procedimientos de revascularización en los pacientes con ICE. Estudios independientes y minuciosos desarrollados en Suecia, Dinamarca y Finlandia indican que el aumento de la disponibilidad y el uso de las intervenciones endovasculares y quirúrgicas ha dado lugar a un descenso significativo de la amputación por ICE. En el Reino Unido, el número de amputaciones mayores ha alcanzado una meseta, lo que podría reflejar el creciente éxito en el rescate de la extremidad; sin embargo, los estudios más antiguos desarrollados en los Estados Unidos no han podido demostrar el beneficio de la revascularización en las tasas de amputación [13].

La idea de que todos los pacientes que requieren una amputación han progresado paulatinamente, desde una claudicación creciente hasta el dolor en reposo, úlceras y, en último término, amputación, es incorrecta. Se ha demostrado que más de la mitad de los pacientes que sufren una amputación mayor por debajo de la rodilla por una enfermedad isquémica no tenían síntomas de isquemia en la pierna de cualquier tipo

en los seis meses anteriores [14]. La incidencia de amputación mayor según los datos obtenidos en poblaciones de gran tamaño o en estudios nacionales varía entre 120 y 500 casos/millón/año. La relación entre las amputaciones por debajo de la rodilla y por encima de la rodilla en encuestas de gran tamaño es cercana a 1:1.

Sólo el 60% de las amputaciones que se producen por debajo de la rodilla se cura por primera intención, el 15% se cura después de procedimientos secundarios y el 15% debe convertirse en una amputación por encima de la rodilla. El 10% de los casos fallece en el perioperatorio.

VASCULOPATÍA CONCURRENTE

Puesto que la EAP, la arteriopatía coronaria (AC) y la arteriopatía cerebral son todas manifestaciones de la aterosclerosis, no resulta sorprendente que las tres afecciones se presenten juntas.

Afectación coronaria

Los estudios sobre la prevalencia de enfermedad cardiovascular en pacientes con EAP demuestran que los antecedentes, la exploración física y el electrocardiograma identifican una prevalencia de AC y arteriopatía cerebral del 40% al 60% en estos pacientes. En el estudio PARTNERS, el 13% de los sujetos estudiados tenía un ITB <0,90 sin AC o arteriopatía cerebral sintomáticas, el 16% tenía AP junto con AC o arteriopatía cerebral sintomáticas y el 24% tenía AC y arteriopatía cerebral sintomáticas con un ITB normal[8]. Al igual que sucede con la AP sintomática, el diagnóstico de AC depende de la sensibilidad de los métodos utilizados. En el entorno de la atención primaria, aproximadamente la mitad de los pacientes diagnosticados de AP también tienen AC y arteriopatía cerebral; es improbable que la prevalencia de AC sea más alta en los pacientes con AP que son derivados al hospital. La extensión de la AC, valorada tanto por la angiografía como por el calcio en las arterias coronarias medido mediante tomografía computerizada, se correlaciona con el ITB. No resulta sorprendente que los pacientes con AC documentada tengan más probabilidades de tener EAP. La prevalencia de AP en pacientes con cardiopatía isquémica varía en cada serie entre el 10% y el 30%. En estudios de autopsias se ha observado que los pacientes que fallecen por infarto de miocardio tienen el doble de probabilidades de tener una estenosis significativa de las arterias ilíaca y carótida que los pacientes que fallecen por otras causas.

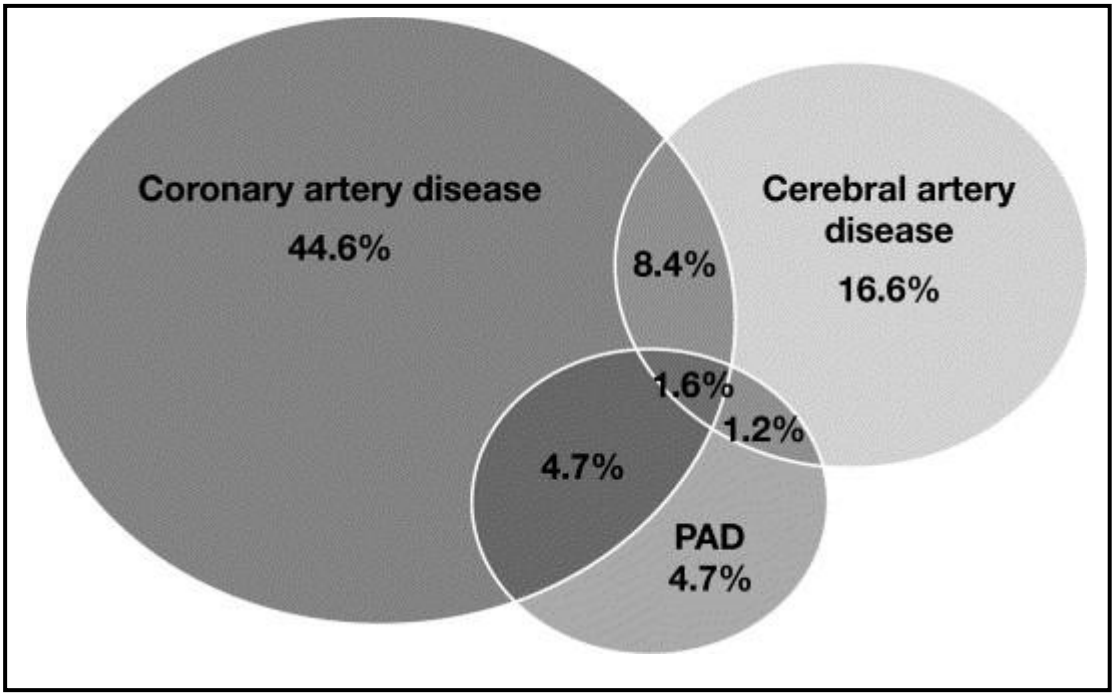


Fig.4. Superposición típica de la enfermedad vascular. TASC II. Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC II). Eur J Vasc Endovasc Surg 2007;33,S1-S75.

Arteriopatía cerebral

La relación entre EAP y arteriopatía cerebral parece ser más débil que con la AC. Mediante el estudio ecográfico Doppler doble se detecta arteriopatía carotídea en el 26%-50% de los pacientes con CI, pero sólo el 5% de los pacientes con AP tendrá antecedentes de episodio cerebrovascular de cualquier tipo. También existe una buena correlación entre el grosor de la íntima en la arteria carótida y el ITB. Existe toda una gama de concurrencias de la enfermedad en las circulaciones cerebral, coronaria y periférica descrita en la literatura. En el estudio REACH (*Reduction of Atherothrombosis for Continued Health*) de pacientes identificados con EAP sintomática, el 4,7% tenía AC concomitante, el 1,2% tenía arteriopatía cerebral concomitante y el 1,6% tenía ambas. Por tanto, aproximadamente el 65% de los casos con AP en esta encuesta mostraba evidencia clínica de otra enfermedad vascular. Sin embargo, en un estudio prospectivo de 1886 en pacientes con 62 años de edad o mayores, sólo el 37% no tenía evidencias de enfermedad en ninguno de los tres territorios [15-16].

Renal

En los estudios también se ha analizado la prevalencia de estenosis de la arteria renal en pacientes con EAP. La prevalencia de estenosis de la arteria renal del 50% o mayor varía entre el 23% y el 42% (compárese esta cifra con la prevalencia de estenosis de la arteria renal en la población general hipertensa, que está en torno al 3%). Aunque no se ha estudiado específicamente, es muy probable que la estenosis de la arteria renal también sea, en parte, un factor de riesgo independiente de mortalidad en pacientes con EAP porque una estenosis de la arteria renal del 50% o mayor se asocia a una tasa de mortalidad 3,3 veces más alta en comparación con la población general [15-18].

EVOLUCIÓN DE LOS PACIENTES

Pacientes con arteriopatía periférica asintomática y con claudicación

El mayor riesgo de episodios cardiovasculares en pacientes con EAP se correlaciona con la gravedad de la enfermedad en las piernas, según determina la medición del ITB. La tasa anual global de episodios cardiovasculares mayores (infarto de miocardio, ictus isquémico y muerte vascular) es aproximadamente del 5%-7%.

Excluyendo aquellos casos con ICE, los pacientes con EAP tienen una incidencia anual del 2%-3% de infarto de miocardio no mortal y su riesgo de angina es entre dos y tres veces mayor que el de la población de edad comparable. Las tasas de morbilidad y mortalidad por todas las causas a 5, 10 y 15 años se acercan al 30%, 50% y 70%, respectivamente. La AC es, con mucho, la causa más frecuente de muerte entre los pacientes con EAP (40%-60%), siendo la arteriopatía cerebral la responsable del 10% al 20% de las muertes. Otros acontecimientos vasculares, principalmente la rotura de un aneurisma aórtico, provocan aproximadamente el 10% de las muertes. Por tanto, sólo el 20%-30% de los pacientes con EAP fallece por causas no cardiovasculares.

Particularmente interesantes son los estudios en los que la diferencia entre las tasas de mortalidad de pacientes con CI y de su población de control de edad comparable no se vio considerablemente alterada a pesar de ajustar los resultados según factores de riesgo como el tabaquismo, hiperlipidemia e hipertensión. Esto resultados sorprendentes, aunque coherentes, sugieren que la presencia de AP indica un grado extenso y grave de aterosclerosis sistémica que es responsable de la mortalidad, con independencia de la presencia de factores de riesgo [19-20].

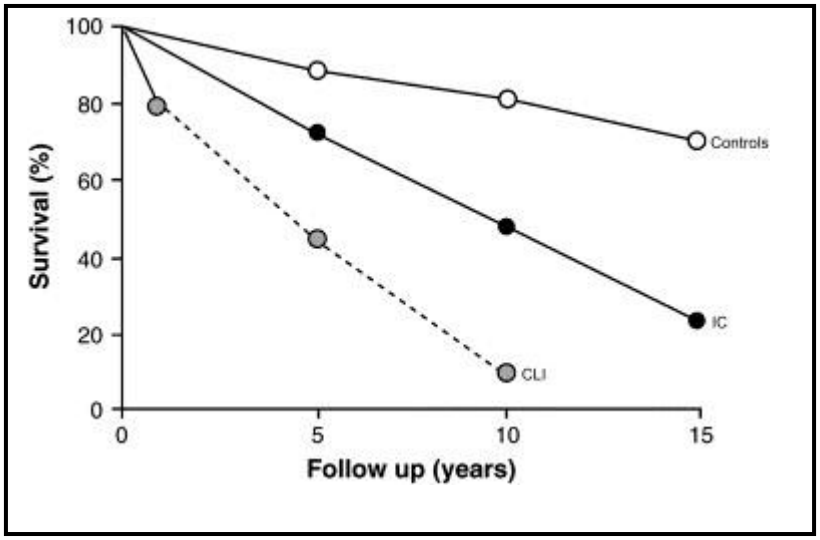


Fig. 5 Supervivencia de los pacientes con enfermedad arterial periférica. TASC II. Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC II). Eur J Vasc Endovasc Surg 2007;33,S1-S75.

Gravedad de la arteriopatía periférica y supervivencia

Los pacientes con ICE crónica tienen una mortalidad del 20% en el primer año tras la presentación y los pocos datos a largo plazo existentes, indican que la mortalidad continúa con la misma tasa. La mortalidad a corto plazo de los pacientes que acuden con isquemia aguda es del 15%-20%. Una vez que han sobrevivido al episodio agudo, su patrón de mortalidad seguirá el de un paciente con claudicación o un paciente con ICE crónica, dependiendo de la evolución del episodio agudo.

Existe una estrecha correlación entre el ITB como medida de la intensidad de la EAP y la mortalidad. Esta relación se ha demostrado en varios estudios en los que se han utilizado distintos valores umbral del ITB. Por ejemplo, en un estudio de casi 2000 sujetos con claudicación, los pacientes con un ITB < 0,50 tenían el doble de mortalidad que los pacientes con claudicación y un ITB > 0,50 en la inclusión. En el *Edinburgh Artery Study* también se ha demostrado que el ITB es un buen factor pronóstico de episodios cardiovasculares mortales y no mortales, así como de la mortalidad total en una población general no seleccionada. Asimismo, se ha propuesto que existe una relación casi lineal entre el ITB y los episodios cardiovasculares mortales y no mortales, asociándose cada descenso de 0,10 del ITB a un incremento del 10% del riesgo relativo de sufrir un episodio vascular mayor [10-22].

CAPÍTULO 2

QUIMIOCINAS: MCP-1

Las quimiocinas son pequeñas proteínas que dirigen la migración de los leucocitos circulantes a los sitios de la inflamación o lesión [23-24-25]. Hay ≈ 50 quimiocinas humanas, que se dividen en cuatro familias en base a las diferencias en su estructura, según la posición relativa de los residuos de cisteína, y función.

La familia más grande que se conoce es la familia de **quimiocinas CC**, en las que los dos primeros de los cuatro residuos de cisteína conservados, característicos de las quimiocinas, son adyacentes entre sí. Las quimiocinas CC tienden a atraer a las células mononucleares y se encuentran en los sitios de inflamación crónica. La quimiocina CC más bien caracterizada es la proteína quimioatrayente de monocitos 1, MCP-1 (también conocida como CCL2), un potente agonista de monocitos, células dendríticas, células T y basófilos.

Se ha descrito que MCP-1 juega un papel clave en el reclutamiento de monocitos de la sangre a las lesiones ateroscleróticas tempranas, el desarrollo de hiperplasia de la íntima después de la angioplastia, así como en la vasculogénesis y la trombosis. Otros miembros de la familia CC incluyen RANTES (CCL5), proteína inflamatoria de macrófagos 1α (MIP-1α, también conocida como CCL3) y MIP-1β (CCL4).

La familia de las **quimiocinas CXC**, de los cuales la IL-8 (CXCL8) es el miembro prototipo, atraen los leucocitos polimorfonucleares y han sido descritas por su implicación en la inflamación pulmonar aguda. IL-8 también activa los monocitos y puede dirigir su reclusión a las lesiones vasculares[26-28]. Las quimiocinas CXC tienen un único residuo de aminoácido entre las dos primeras cisteínas canónica.

La tercera familia, **la familia CX3C**, sólo tiene una citoquina conocida por el momento, la fractalquina (FK, o CX3CL1). FK consiste en un dominio de quimiocinas soluble unido a un tallo tipo mucina y un dominio transmembrana [29-30]. Por lo tanto, a diferencia de otras quimiocinas soluble, es una proteína transmembrana de tipo 1. En su larga estructura, unida a la membrana de proteínas, FK es un medio eficiente de adhesión celular del receptor que puede detener las células bajo condiciones de flujo fisiológico[31-32] . FK puede disociarse de la membrana celular por el factor de necrosis tumoral α de conversión enzimático y por la metaloproteasa ADAM-10 para liberar una proteína soluble. De esta forma soluble, FK es un quimioatrayente potente de monocitos, células T y células asesinas naturales (NK)[33]. De este modo, dependiendo de si existe como una proteína inmovilizada o una proteína soluble, FK puede

funcionar como un receptor de adhesión celular o como un quimioatrayente. FK se expresa en las lesiones ateroscleróticas y tiene varias posibles funciones en la aterogénesis. CXCL16 también tiene un dominio soluble relacionado con un tallo de mucina[34] . CXCL16 se expresa en los macrófagos y células dendríticas, y es de especial relevancia en las enfermedades cardiovasculares, encargándose de recoger los lípidos oxidados[35] .

El último grupo es la familia de **quimiocinas limfotácticas C**, donde únicamente hay 2 residuos de cisteína en la proteína madura.

Las quimioquinas ejercen sus efectos mediante la activación celular de receptores transmembrana de dominio G acoplados a proteínas. La respuesta de un leucocito a una determinada quimioquina está condicionada por su dotación de receptores de quimioquinas. La unión de las quimiocinas activa una cascada de transducción de señales que activa la fosfatidilinositol-3 quinasa, aumenta los niveles de inositol trifosfato y de calcio intracelular, activa Rho y las proteínas mitogénicas-activadas quinasas, y finalmente estimula el cambio de forma de la actina, estimulando el movimiento de las células. Aunque todavía no se entienden completamente, las vías de señalización que conducen a la quimiotaxis se basan en Gαi como el vínculo inicial con el receptor activado y parece ser dependiente de la activación de uno o más isoformas de la fosfatidilinositol 3-quinasa[36-37].

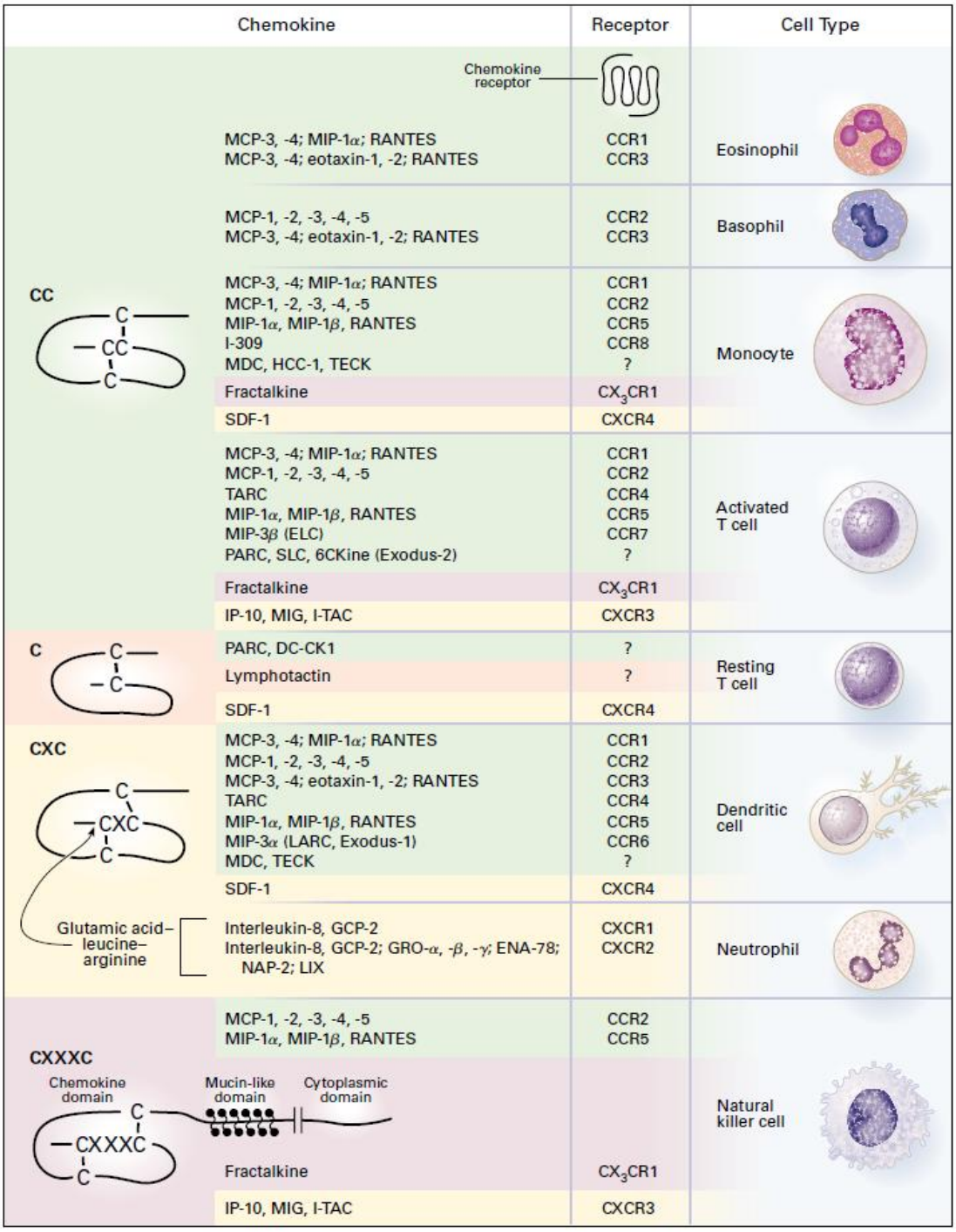


Fig. 6. Las quimiocinas y sus receptores.

Las quimiocinas son proteínas homólogas de 8 a 10 kd que se subdividen en las familias sobre la base de la posición relativa de los residuos de cisteína. En las α-Quimiocinas, los dos primeros residuos de cisteína están separados por un solo aminoácido ácido (CXC), mientras que en las β-Quimiocinas, las dos primeras cisteínas de los residuos son adyacentes entre sí (CC). Las α-Quimiocinas que contienen la secuencia de ácido glutámico-leucina-arginina anterior a la secuencia de CXC son quimiotácticas para neutrófilos, y los que no contienen esta secuencia actúan sobre los linfocitos. La quimiocina linfotactina C sólo tiene dos cisteínas, y las quimiocinas CXXXC tiene tres aminoácidos ácidos que separan las dos primeras cisteínas.
Los receptores de quimiocinas están acoplados a proteínas G-proteínas que se expresan en los subgrupos de los leucocitos. Cuatro receptores de quimiocinas CXC humana , ocho receptores de quimioquinas CC humanos, y un receptor humano de quimioquinas CXXXC han sido identificados.

La arteriosclerosis.

Las estrías grasas, la grasa característica de las primeras lesiones ateroscleróticas, están compuestas de macrófagos cargados de lípidos llamados células espumosas. Los estudios realizados en cerdos y primates[38-39] indican que los monocitos circulantes en sangre son los precursores de estas células espumosas. Varias líneas de evidencia apoyan ahora la hipótesis de que MCP-1 juega un papel fundamental en el reclutamiento de monocitos en las lesiones tempranas. Los primeros estudios informaron que MCP-1 está presente en los macrófagos ricos en placas ateroscleróticas en los seres humanos y los primates[40-41]. Los lípidos oxidados han sido descritos como mediadores de la aterosclerosis y la formación de células espumosas[42]. Los estudios de Cushing et al[43] demostraron que las lipoproteínas de baja densidad mínimamente oxidadas, inducían la producción de MCP-1 en las células de la pared vascular. MCP-1 por lo tanto surgió como un posible vínculo molecular entre las lipoproteínas oxidadas y el reclutamiento de células espumosas en la pared del vaso.

Los estudios de ratones transgénicos que sobreexpresan MCP-1 y de ratones deficientes en MCP-1, o su receptor, proporcionan pruebas de que la MCP-1 trabaja a favor del reclutamiento de monocitos al ateroma. Por lo tanto, la sobreexpresión de MCP-1 en tejidos específicos provoca una infiltración localizada de los monocitos / macrófagos[44]. En los estudios de trasplante de médula ósea, la sobreexpresión de MCP-1 en macrófagos en la pared del vaso llevó a una mayor formación de células espumosas y al aumento de la aterosclerosis.

CCR2 es el único receptor establecido funcional para MCP-1 en células hematopoyéticas, y su supresión en los ratones deficientes en apolipoproteina E (apoE), ofrece una protección significativa de la acumulación de macrófagos y de la formación de las lesiones ateroscleróticas en respuesta a una dieta rica en grasas [45-48].

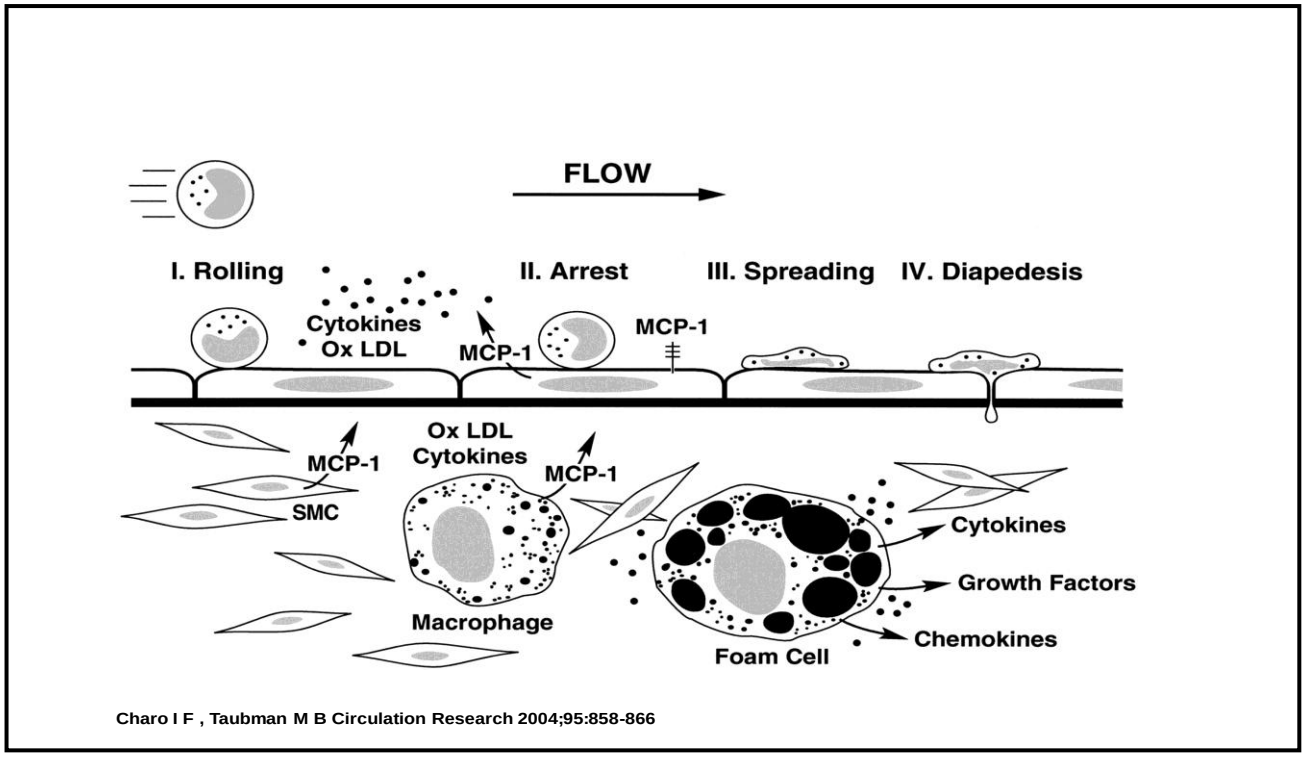


Figura 7: Los monocitos circulantes en sangre se dirigen al endotelioo. MCP-1 y otras quimiocinas son sintetizadas por las células endoteliales, células del músculo liso y los macrófagos en respuesta a los lípidos oxidados. Cómo MCP-1 se localiza en el endotelio, se desconoce, pero puede implicar la unión a los proteoglicanos sulfato de heparina. Después de entrar en el espacio subendotelial, los monocitos se diferencian en macrófagos. La ingestión continua de lípidos conduce a la formación de células espumosas, y tanto los macrófagos como las células espumosas continúan secretando moléculas bioactivas, tales como factores de crecimiento y quimioquinas, que pueden reclutar y activar los monocitos adicionales.

Dipòsit Legal: T. 191-2013

CAPÍTULO 3

PARAOXONASAS

La familia de las paraoxonasas (PON) contiene tres componentes (PON1, PON2 y PON3), que presentan propiedades antioxidantes. Últimamente ha habido un mayor estudio e investigación de las funciones de PON2 y PON3, pero PON1 sigue siendo con mucho el más estudiado de la tres enzimas.

PON1 es una esterasa calcio-dependiente que fue descrita por primera vez por su capacidad para hidrolizar organofosfatos y pesticidas, paraoxón incluidos, que inspiró el nombre de las tres enzimas.

PON1 es una glicoproteína de 43-45 kDa, expresada en varios tejidos[49], pero se sintetiza principalmente en el hígado y circula dentro de las lipoproteína de alta densidad (HDL) [50].

PON1 ha sido objeto de investigación más intensa, debido a su evidente capacidad para proteger las lipoproteínas de baja densidad (LDL) contra el estrés oxidativo, reducir la formación de células espumosas y prevenir el desarrollo de la aterosclerosis[51]. Los polimorfismos del gen de la PON1 se han asociado con varias enfermedades humanas, incluyendo las enfermedades coronarias[52], la enfermedad de Parkinson[53] , la diabetes tipo 2[54] y enfermedad inflamatoria intestinal[55].

A pesar del interés creciente en PON2, todavía hay poca información sobre sus características y sus funciones. A pesar de que este miembro de la familia de las PON no se asocia con las partículas de HDL circulantes, se le ha implicado en la reducción del estrés oxidativo y en la protección contra la arterioesclerosis[56]. PON2 se expresa en casi todos los tejidos humanos, incluyendo los pulmones, el hígado, el corazón y intestino[57]. En las células vasculares, PON2 aparece en dos isoformas glucosiladas de aproximadamente 40-43 kDa[58] y sus polimorfismos genéticos han sido implicado varias enfermedades, como las enfermedad cardiovasculares [59-60], la diabetes tipo 2 [61-62] y las enfermedades inflamatorias del intestino[63].

PON3,es similar a PON1 en términos de expresión, función y ubicación. Ambos son capaces de retrasar la oxidación in vitro de LDL, siendo PON1 más eficaz que PON3 a este respecto[64]. Hay pocos datos disponibles hasta el momento de los polimorfismos de la PON3, y los efectos de estas variantes en las enfermedades humanas aún no se conocen[65].

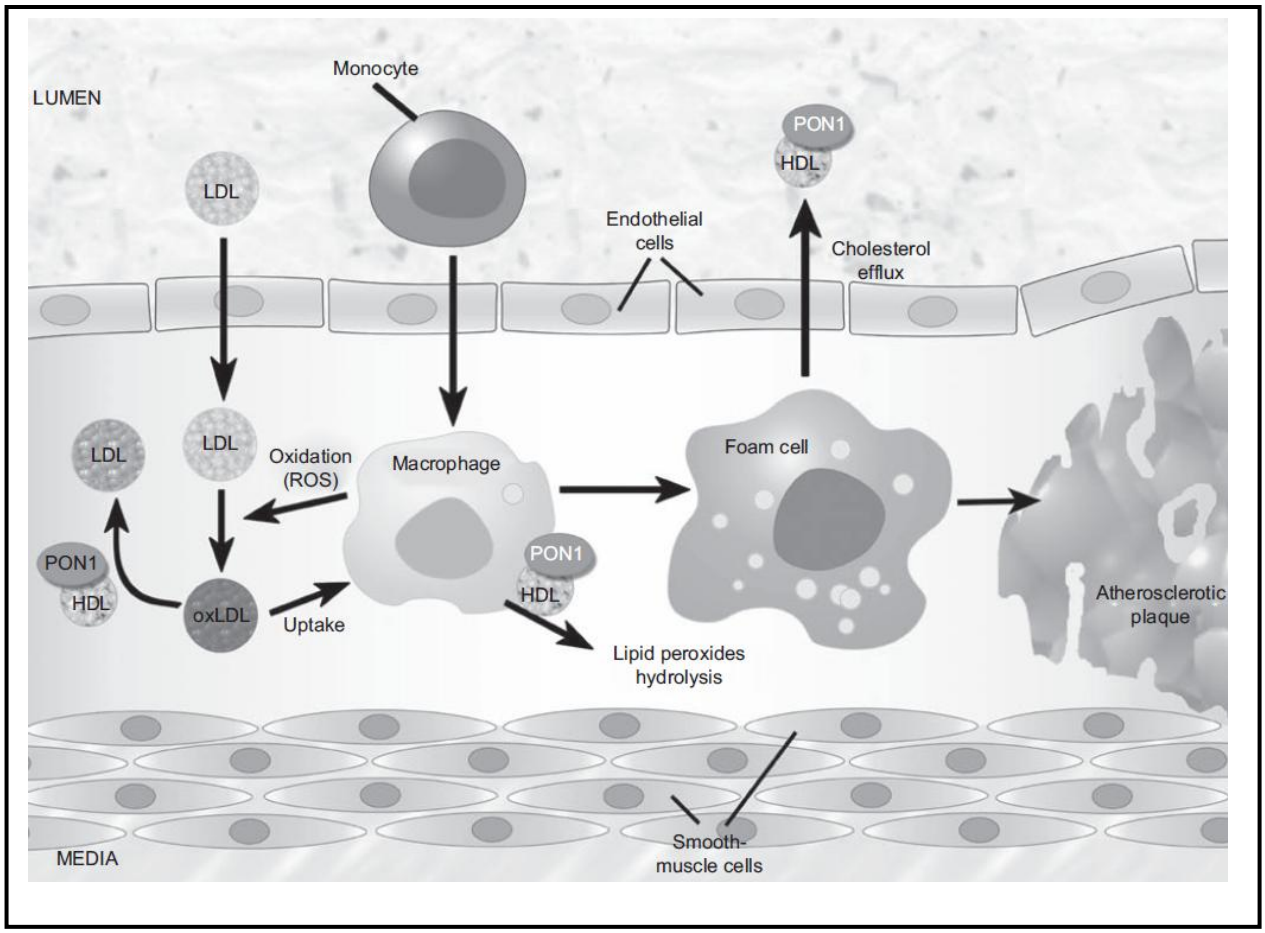


Fig. 8. Esquema del inicio de la arteriosclerosis y la función protectora de PON1.

Los monocitos circulantes se activan,como consecuencia de un entorno pro-oxidativo y proinflamatorio, y se diferencian en macrófagos. Las partículas de LDL también atraviesan la pared. Los macrófagos promueven la oxidación de LDL mediante la liberación de radicales libres. Las LDL oxidadadas entran en los macrófagos y contribuyen a su conversión a células espumosas.La oxidación de LDL y la formación de células espumosas son las señas de identidad de la aterogénesis precoz. La hidrólisis de PON1 de los lípidos oxidados en LDL revierte esta lipoproteína en LDL normal y, por tanto, atenúa el desarrollo de la arteriosclerosis. Camps et al. Critical Reviews in Clinical Laboratory Science, 2009; 46(2): 83-106.

Las tres formas de PON son protagonistas en el mantenimiento de un bajo nivel en el estado oxidativo de la circulación y, por tanto, en la prevención de la aterosclerosis. Las asociaciones de sus polimorfismos con varias enfermedades humanas muestran una posible implicación de estos enzimas en otros órganos. Es de destacar que los polimorfismos genéticos de PON1 se ha demostrado que representan más del 60% de las variaciones interindividual en la concentración de la enzima y su actividad, pero las tres PON han demostrado ser moduladas por diversas moléculas nutricionales y farmacológicas y algunos eventos fisiopatológicos como la inflamación y el estrés oxidativo.

PON1, HDL E INFLAMACIÓN

Gran cantidad de datos experimentales muestran un vínculo entre la la peroxidación lipídica y la inflamación[66]. Las partículas de HDL han demostrado que poseen propiedades antioxidantes[67-69] y antiinflamatorias, incluyendo la supresión de las moléculas de adhesión celular inducidas por citoquinas (CAM)[70-72]. Estudios recientes realizados en voluntarios sanos mostraron una estrecha relación entre los niveles de HDL, la respuesta inflamatoria de la endotoxina, la incidencia y severidad de los síntomas clínicos y las concentraciones plasmáticas de factor de necrosis tumoral así como las interleucinas 1, 6 y 8, y la proteína quimioatrayente de monocitos-1 (MCP-1), siendo mayor en los sujetos con bajas concentraciones de HDL que aquellos con normalidad en los niveles de HDL[73] .

MCP-1 está íntimamente involucrado en la respuesta inflamatoria. Las quimiocinas regulan la migración de monocitos en los tejidos y su posterior diferenciación en los macrófagos [74].

En un sistema de co-cultivo de monocitos humanos y de células endoteliales, la HDL atenúa la estimulación de la migración de monocitos inducida por las LDL oxidadas[75], proporcionando evidencia indirecta de que el HDL elimina la producción de MCP-1. Desde que la PON1 actúa como un enzima antioxidante, no sería descabellado considerar que participa en estas funciones antiinflamatorias de HDL[76].

Mackness et al.[77] demostraron por primera vez que la PON1 inhibe la producción de MCP-1 en las células endoteliales incubadas con LDL oxidado. Estos autores hallaron que el colesterol HDL, así como la PON1 recombinante, suprimen la producción de MCP-1 en cultivos de células endoteliales, mientras que el HDL aviar (que, a diferencia de el HDL humano, no tiene PON1 vinculado con él) no pudo provocar esta reacción.

La inhibición de PON1 sobre la producción de MCP-1 parece ser debida a su capacidad para inhibir la oxidación de LDL.

Estudios en animales de experimentación apoyan el concepto de una función antiinflamatoria de la PON1. Los ratones transgénicos (generados por la microinyección de genes clonados de PON1 humana en óvulos fecundados) alimentados con una dieta rica en grasas y rica en colesterol, desarrollaron menores lesiones ateroscleróticas, menor estrés oxidativo, y aproximadamente una expresión un 44% menor en sus aortas en comparación con las aortas de ratones no tratados[78]. Estos resultados confirman otros estudios que muestran que la PON1 de ratones tenían mayor peroxidación de lípidos periféricos y un mayor grado de estrés oxidativo de macrófagos[79]. Las investigaciones posteriores han demostrado que esta función antioxidante y antiinflamatorios de PON1 es compleja y puede involucrar no sólo a una la inhibición de la peroxidación lipídica, sino también un aumento de la macrófagos a través de la salida del colesterol HDL.

Parece claro que la PON1 inhibe la transmigración de monocitos a macrófagos inducida por el estrés oxidativo, debido a su capacidad de degradar los peróxidos lipídicos y a la regulación a la baja de la producción de MCP-1 por las células endoteliales vasculares, así como a su capacidad para aumentar los macrófagos asociados a colesterol. Este razonamiento proporciona un mecanismo de vinculación entre la peroxidación lipídica, la inflamación, y las funciones de PON1 y HDL.

La respuesta de fase aguda asociada con muchas enfermedades infecciosas e inflamatorias pueden estar relacionados a una disminución en la actividad de PON1 en suero y, en consecuencia, a la protección más ineficaz contra el estrés oxidativo, lo que agrava el proceso inflamatorio.

La molécula de HDL constituye el medio natural para la actividad de la enzima PON1, y la actividad es sensible a alteraciones en las partículas de HDL. Incluso con HDL normal, la PON1 activa se encuentra en una parte muy específica de la población de HDL, es decir, la subfracción menos densa de HDL_3, que contiene las apolipoproteínas E y J[80-81]. La PON1 necesita de la apolipoproteína A-I para su actividad. Por el contrario, la presencia de la apolipoproteína A-II en el colesterol HDL está asociado con la inactivación PON1[82]. Además, la fase aguda de HDL, no sólo inactiva las ya sintetizadas moléculas de PON1, sinó que además también inhibe la expresión genética hepática [83-85].

PON2 Y PON3

En la actualidad, no se sabe mucho acerca de la PON2 PON3. Sus genes fueron identificados en 1996, cuando Primo-Parmo et al.[49] identificaron un gran número de secuencias de cDNA en la Base de datos del genoma con una homología significativa, pero no idénticas, con las PON1 humanas. El porcentaje de identidad entre los genes PON1, PON2 y PON3 es similar (70%) y se cree que los genes derivan de un mismo precursor[86].

Pronto se hizo evidente que PON2 participa en la regulación del metabolismo de las lipoproteínas. Boright et al.[87] demostraron que los polimorfismos genéticos de PON2 están asociados con variaciones en el colesterol sérico y las concentraciones de apolipoproteína A-I. Casi al mismo tiempo, Sanghera et al.[88] mostró que los polimorfismos de PON2 estaban relacionados con las enfermedades cardiovasculares. Ng et al.[55] demostró que las PON2 no están presentes en las lipoproteínas, aunque su expresión genética se ha detectado en varios tejidos humanos. También se informó que las células transfectadas con el gen humano PON2 tienen mayor capacidad antioxidante y son menos eficaces en la oxidación de LDL que las células que no fueron transfectadas.

PON3 está presente en las HDL y previene LDL de la oxidación in vitro. Ambos son capaces de hidrolizar lactonas, pero no paraxón ni otros xenobióticos [89-91].

Dipòsit Legal: T. 191-2013

II *HIPÓTESIS Y OBJETIVOS*

Dipòsit Legal: T. 191-2013

HIPÓTESIS

El presente proyecto tiene como finalidad investigar la utilidad e importancia de nuevos marcadores de stress oxidativo e inflamación en el diagnóstico, pronóstico y seguimiento de la enfermedad arterial periférica (EAP).

OBJETIVOS

1. Búsqueda de nuevos marcadores para el diagnóstico y seguimiento en pacientes con EAP, basados en la determinación de parámetros relacionados con el stress oxidativo y la inflamación, fenómenos que juegan un papel fundamental en el desarrollo de la arteriosclerosis.

2. Proponer una nueva aproximación bioquímica basada en el papel coordinado entre las paraoxonasas (PON1) y la proteína quimioatrayente de monocitos (MCP-1), moléculas que intervienen en la regulación del stress oxidativo y los procesos inflamatorios.

3. Determinar la posible asociación entre la concentración de PON3 circulante y sus polimorfismos genéticos con la enfermedad arterial periférica (EAP) y la enfermedad coronaria.

III *RESULTADOS*

Dipòsit Legal: T. 191-2013

ESTUDIO 1

The role of combined assessment of defense against oxidative stress and inflammation in the evaluation of peripheral arterial disease

Current molecular medicine 2011, 11, 453-464

Los factores que lesionan el endotelio generan una respuesta inflamatoria que es el inicio del proceso de la enfermedad arteriosclerótica.

La arteriosclerosis en la enfermedad arterial periférica en pacientes sintomáticos, afecta ampliamente al territorio arterial de las extremidades inferiores. La respuesta inflamatoria es extrema en estos pacientes, representando un proceso inflamatorio de gran importancia que puede medirse mediante variables relacionadas con la inflamación. Creemos que la interacción entre la defensa frente el estrés oxidativo y la inflamación, PON1 y MCP-1, puede ser útil para valorar esta respuesta inflamatoria.

Para ello, se han medido los valores de MCP-1 y de PON1 (y parámetros relacionados) en un grupo de pacientes con EAP sintomáticos y dos grupos más, uno con pacientes sanos, y otro grupo de pacientes afectados de cardiopatía isquémica sin presentar EAP. Hemos observado que los valores para MCP-1 y PON1, especialmente su concentración, diferencia casi perfectamente pacientes de controles, sugiriendo, así pues que, existe un equilibrio entre MCP-1 y PON1 que puede ser detectado en sangre y que puede actuar como un indicador de la extensión de la arteriosclerosis o del daño endotelial.

Hemos encontrado que los resultados obtenidos de MCP-1 y la concentración de PON1, así como PON1 paraoxonasa y actividades lactonasa, prueban nuestra hipótesis y pueden constituir un indicador del estado de enfermedad que pueden actuar como biomarcadores. La disminución de las actividades paraoxonasa y lactonasa se asociaron significativamente con el aumento de las concentraciones circulantes de MCP-1. Un aumento de MCP-1 se asocia con una disminución de la concentración de PON1, diferenciando claramente los pacientes con respecto a los controles y mostrando un fuerte valor predictivo.

Hemos mostrado una buena correlación de MCP-1 con los niveles plasmáticos de β2 microglobulina (β2M) y proteína C reactiva (PCR), por lo tanto, proponemos el uso combinado de ambos.

Los resultados en los pacientes con CAD, con posiblemente una menor carga de aterosclerosis, también sugiere un papel de coordinación entre PON1 y MCP-1.

En conclusión, nuestros resultados clínicamente identifican una relación inversa entre MCP-1 y PON1, que es independiente de las variaciones genéticas y que la relación

entre la defensa frente el estrés oxidativo y la inflamación puede ser detectado mediante simples mediciones de laboratorio en pacientes con aterosclerosis.

The Role of Combined Assessment of Defense Against Oxidative Stress and Inflammation in the Evaluation of Peripheral Arterial Disease

A. Rull[1], R. García[2], L. Fernández-Sender[1], R. Beltrán-Debón[1], G. Aragonès[1], J.M. Alegret[3], C. Alonso-Villaverde[4], B. Mackness[1], M. Mackness[1], J. Camps[1], V. Martin-Paredero[2] and J. Joven*[,1]

[1]*Centre de Recerca Biomèdica, IISPV, Universitat Rovira i Virgili, Spain;* [2]*Servei d'Angiologia, Cirurgia Vascular i Endovascular, Hospital Universitari Joan XXIII de Tarragona, Universitat Rovira i Virgili, Spain;* [3]*Cardiology Unit, Hospital de Sant Joan. Reus, Spain;* [4]*Servei de Medicina Interna. Hospital Son Llàtzer, Palma, Mallorca, Spain*

Abstract: Atherosclerosis in symptomatic peripheral arterial disease affects wide portions of numerous arteries in lower extremities. The resulting active inflammation in a considerable amount of arterial tissue facilitates systemic detection *via* measurement of inflammation-related variables. We reasoned that the combined assessment of defense against oxidative stress, in the form of paraoxonase-1 (PON1), and monocyte migration measured as circulating (C-C motif) ligand 2 (CCL2), may play a role in the evaluation of these patients. Plasma CCL2 and serum PON1-related variables, assessed by their interaction with functional genetic variants, were measured in a cross-sectional study in patients with symptomatic PAD. We found that PON1 activity and concentration were significantly lower and CCL2 concentration higher in PAD patients compared to controls, that the combination of plasma CCL2 and PON1- related values, especially PON1 concentration differentiated, almost perfectly, controls from patients and that the expression of CCL2 and PON1 generally co-localized in the atherosclerotic lesion. Since no association with genetic variants was found, such a relationship is probably the result of the disease. Our data suggest a coordinated role between CCL2 and PON1 that may be detected in blood with simple measurements and may represent an indicator of the extent of atherosclerosis.

Keywords: Atherosclerosis, CCL2, inflammation, lactonase, monocyte chemoattractant protein-1, paraoxonase-1, peripheral vascular disease, polymorphism.

1. INTRODUCTION

Generalized atherosclerosis is likely in symptomatic peripheral arterial disease (PAD), affecting wide portions of numerous arteries in the lower extremities. This is usually the effect of a sustained and silent progression of the disease in which adequate and effective prevention measures are either applied too late or not implemented at all [1-6]. Conceivably, the burden of atherosclerosis is higher than in those patients with adverse events in other arterial territories and this may represent a differential aspect of this disease that may aid in designing strategies to facilitate prompt identification. For instance, the fact that inflammation is present in a significant amount of arterial tissue facilitates its systemic detection *via* routine laboratory tests, especially high-sensitivity C-reactive protein (hsCRP) and β_2-microglobulin (β2M) [7-10]. Searching for potential indicators of disease state and/or susceptibility, we propose a novel approach based on a putative coordinated role between paraoxonase-1 (PON1) and monocyte chemoattractant protein-1/chemokine (C-C motif) ligand 2 (CCL2), that is the combined value of markers of a plausible interaction between defense against oxidative stress and inflammation.

Cytokines are involved in essentially every important biologic process, especially inflammation, and represent feasible therapeutic targets [11]. Chemokines, CCL2 in particular, are central to the vascular inflammatory response as mediators of monocyte recruitment into the arterial wall [12]. We have previously found *in vitro* that PON1 inhibits the production of CCL2 induced by oxidative stress in endothelial cells and that both PON1 and CCL2 are ubiquitously distributed in mouse tissues suggesting a joint localization and combined systemic effects [13-14]. Clinical data suggest that circulating CCL2 concentration or serum PON1 esterase and lactonase activities, or both, may be important biomarkers of a variety of diseases involving inflammatory response to an increased oxidative stress [15-20]. However, to the best of our knowledge, the hypothesis that an inverse relationship may be detected in blood with simple measurements and may represent an indicator of the extent of atherosclerosis is novel. With the rationale that relationships, if any, will be more evident in patients with a high burden of diseased arteries we have performed a cross-sectional study in patients with

*Address correspondence to this author at the Centre de Recerca Biomèdica, IISPV, Universitat Rovira i Virgili. Carrer Sant Llorenç 21, 43201, Reus, Spain; Tel: +34977310300; Fax: +34977342105; E-mail: jorge.joven@urv.cat

1566-5240/11 $58.00+.00

symptomatic PAD to explore whether genetic and biochemical assessment of CCL2 and PON1 may add substantial information to the pathogenesis of advanced atherosclerosis. Further, the balance between CCL2 and PON1 may affect the efficacy of frequently prescribed medications in these patients [21, 22].

2. MATERIALS AND METHODS

2.1. Experimental Design

The study was approved by the institutional review committee and participants signed fully-informed consent. Patients, all males and aged between 55 and 80 years, were recruited consecutively between 2008 and 2009 among patients attending our outpatient Clinic in which clinically diagnosed symptomatic PAD was considered to require hospitalization for further evaluation and/or surgical procedures. At entrance, relevant data were collated by clinical records, interview and physical examination. Participants were considered to be current smokers if they have been smoking within the previous year and former smokers if consumption of tobacco products has ceased for at least one year. Data on alcohol beverage consumption was assessed by the number of standard drink units per day and per week. Diabetes was defined by history, use of diabetes medication, or fasting serum glucose levels $\geq$126mg/dL (6.99 mmol/L). Hypertension was defined as a systolic blood pressure $\geq$ 140 mmHg, diastolic blood pressure $\geq$ 90 mmHg, previous physician's diagnosis or medication use. Dyslipidemia was considered to be present by previous diagnosis, medication use or high levels of LDL-cholesterol ($\geq$ 160 mg/dL or 4.13 mmol/L) and/or low levels of HDL-cholesterol ($\leq$ 40mg/dL or 1.03 mmol/L). Selected laboratory parameters were measured as well as the ankle to brachial index (ABI) and the degree of internal carotid artery (ICA) stenosis using widely accepted methods and recommendations [23, 24]. The degree of ICA was classified using the duplex criteria developed by the University of Washington based on the percentage of arterial diameter reduction. We finally included 85 patients once excluded those in which there were signs of infection (n=35), renal disturbances (n=9), liver disease (n=9), neoplasia (n=3) or autoimmune diseases (n=15). For further analyses, patients were considered to have moderate (ABI <0.9 and >0.4) or severe (ABI<0.4) disease according to previously published criteria [23]. For genotypic and laboratory comparisons, a group of sex, weight and age-matched ostensibly healthy individuals with ABI ≥ 1.0 was further recruited (n=170). To further explore the hypothesis concerning the extent of atherosclerosis we selected samples and data from an open access collection (CARDIOBANC) of patients who survived a myocardial infarction and had well documented coronary artery disease (CAD). They were male (n =85) matched with PAD patients for body mass index (BMI), incidence of diabetes, hypertension and corresponding treatments as well as for percentage of current-past smokers. In these patients, there were no significant alterations in ICA and ABI measurements were >1.0. They also were significantly younger (49.12 ± 0.81 years) suggesting a probably lower burden of atherosclerosis.

2.2. Laboratory Measurements

Fasting blood samples were drawn. Standard measurements were performed immediately in an LXi 725-Synchron (Beckman Coulter, Fullerton, California, USA) automatic analyzer using enzymatic assays or chemiluminescent immunoassays, including β2M and hsCRP using reagents for turbidimetry from BioKit (Barcelona, Spain). Insulin resistance was estimated by using the homeostasis model assessment index (HOMA-IR). Additional plasma and serum aliquots were stored at -80º C until analyses. Plasma concentration of CCL2, interleukin-6 (IL-6), interleukin-8 (IL-8), soluble CD40 ligand (sCD40L), soluble P-Selectin (sP-Selectin) and tissue plasminogen activator (t-PA) were measured with a commercially multiple enzyme-linked assay (FlowCytomix, Bender MedSystems). Serum paraoxonase and lactonase activities, as well as serum PON1 concentration were measured as described [18, 19]. Samples from collection were stored at -80º C between 1 and 5 years but possible measurement errors are considered negligible [21]. To confirm the presence of CCL2 and PON1 in atherosclerotic lesions, we performed immunohistochemical analyses in available arterial tissues (n=20) essentially as described [25]. For genotyping, DNA was extracted from EDTA blood using a column-based method from Qiagen (IZASA, Barcelona, Spain). We selected 6 common CCL2 single-nucleotide polymorphisms (SNPs) and 7 PON1 SNPs spanning the genes according to published data [26, 27]. Details on primers and SNP identification appear at Supportive/Supplementary Table **1**.

2.3. Statistical Analysis

Sample size was calculated with Open Epi [28] in an exposed to unexposed ratio of 1:2 using data from previously accepted biomarkers. Kolmogorov-Smirnov test was used to check for normality of distribution of the variables. Selected variables are presented as mean (SEM) unless otherwise stated. Comparisons between groups were made with Student's unpaired *t* test or Kruskal–Wallis one-way analysis of variance. For the multiple correlation analyses, data was positively skewed using natural logarithm. The Hardy-Weinberg equilibrium (HWE) and allele frequencies were assessed and compared using chi-square test. The effect of each SNP in PAD was assessed using dominant, co-dominant and recessive models and sample size was considered to be adequate to detect modest effects according to the allele frequency and sample sizes previously reported [26, 27]. To evaluate haplotype blocks, linkage disequilibrium (LD) between loci expressing genetic variation and pairwise measurements (D' and r2) the Haploview software

package was used [29]. To analyze the association between variables, multiple regression models were used. Data, including odds ratios and receiving operating characteristic (ROC) curves were analyzed with SPSS, version 18.0 (SPSS Inc., Chicago III).

3. RESULTS

3.1. The Effect of ABI Values on Outcomes of Patients with PAD: Clinical Characteristics

All patients were symptomatic and according to the Fontaine classification, most were included in grades III and IV. Moderate claudication was observed in patients with moderate disease according to the ABI values (Table **1**). There were no obese (BMI>30) patients but overweight (BMI>25) was common and had no apparent effect on the severity of the disease. As expected, patients were aged and the incidence of current or past smoking habit, diabetes mellitus and dyslipidemia was considerably higher than that observed in the general population but these were not significant factors with respect to the severity of the disease. Accepted treatment to correct cardiovascular risk factors were prescribed in most patients. Hypertension and the consequent use of anti-hypertensive agents were clearly higher among patients with severe PAD. Antecedents of coronary heart disease were recorded in 1 out of 5 patients but cerebrovascular events were not recorded. This is particularly important because all patients showed concomitant carotid disease, most with a 16 to 49% reduction in the diameter of ICA. No differences were found between right and left ICA examinations (Supportive/Supplementary Table **2**). Surprisingly, severe cases (>80%) of stenosis were only observed among patients with moderate PAD. The severity of PAD also segregated patients with respect to the final treatment outcome after evaluation (Supportive/ Supplementary Table **3**); there were no differences in endovascular treatment, stenting or amputation but patients with moderate PAD received more medical treatment and less bypass surgery than patients with severe PAD.

3.2. Selected Laboratory Measurements were not Significantly Affected by ABI Values but Discriminate with Respect to Unaffected Controls

All selected variables, with the exception of plasma t-PA, showed significant changes in patients with respect to those obtained in the control group (Table **2**). Plasma concentration of inflammation- and PON-related variables were consistently increased or decreased respectively in patients with respect to controls and associated with HDL-cholesterol levels.

Table 1. Selected Clinical Variables in Patients with Lower Extremity Peripheral Artery Disease (PAD) and Segregated According to ABI Values

	Total PAD patients n=85	Moderate PAD n=54	Severe PAD n=31	p-value
Clinical characteristics, %				
Age, years	69.61 ± 0.93	70.22 ± 1.14	68.54 ± 1.61	0.392
BMI, Kg/m^2	24.98 ± 0.37	25.14 ± 0.45	24.70 ± 0.64	0.572
Current + past smokers	75.2	77.8	87.1	0.290
Alcoholism	8.9	11.1	6.5	0.479
Diabetes	59.4	57.4	58.1	0.953
Hypertension	62.4	53.7	74.2	0.062
Dyslipidemia	30.7	27.8	38.7	0.297
Coronary heart disease	21.8	18.5	29.0	0.263
Medication, %				
Lipid-lowering treatment	36.5	33.3	41.9	0.428
Diabetes treatment	39.6	32.3	40.7	0.437
ACE/ARB	30.6	25.9	38.7	0.218
Beta-blockers	9.4	9.3	9.7	0.949
Other anti-hypertensive	42.4	33.3	58.1	0.026
Anti-platelet agents	67.1	61.1	77.4	0.124
Pentoxiphiline and/or cilostazol	14.9	16.7	16.1	0.949
Fontaine classification, %				
IIb, moderate to severe claudication	9.4	14.8	0.0	0.016
III, ischemic rest pain	40.0	37.0	45.2	0.462
IV, ulceration or gangrene	49.4	46.3	54.8	0.448

Moderate PAD defined by ABI between 0.40-0.90. Severe PAD defined by ABI <0.40.
Age and BMI are given as mean ± SEM.
ACE: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker.

Table 2. Laboratory Measurements in Patients with Peripheral Artery Disease (PAD) as Compared with those Obtained in Unaffected Controls

	Controls (n=170)	Total PAD patients (n=85)		p-value
Hemogram				
Red Blood Cells ($x10^{12}$/L)	4.98 (0.04)	4.15 (0.07)	↓	<0.001
Hemoglobin (g/dL)	14.98 (0.14)	12.36 (0.21)	↓	<0.001
Hematocrit (%)	44.63 (0.43)	37.88 (0.65)	↓	<0.001
Leukocytes ($x10^9$/L)	6.68 (0.14)	8.46 (0.30)	↑	<0.001
Platelets ($x10^9$/L)	217.38 (5.37)	266.62 (10.40)	↑	<0.001
Biochemical variables				
Total cholesterol, mmol/L	5.46 (0.90)	3.85 (0.09)	↓	<0.001
HDL-cholesterol, mmol/L	1.40 (0.32)	1.04 (0.03)	↓	<0.001
LDL-cholesterol, mmol/L	3.33 (0.09)	2.20 (0.09)	↓	<0.001
Triglycerides, mmol/L	1.61 (0.14)	2.90 (0.09)	↑	<0.001
Glucose, mmol/L	5.13 (0.14)	6.49 (0.26)	↑	<0.001
Insulin, pmol/L	49.83 (3.10)	138.70 (22.60)	↑	0.002
HOMA-IR	1.64 (0.12)	6.55 (1.45)	↑	0.001
Inflammation-related measurements				
β2M (mg/L)	0.9 (0.4)	4.15 (0.28)	↑	<0.001
CCL2 (pg/mL)	427.91 (18.02)	645.11 (27.49)	↑	<0.001
High-sensitivity CRP (mg/L)	2.30 (0.30)	23.01 (2.67)	↑	<0.001
Interleukin-6 (pg/mL)	3.29 (0.98)	22.35 (2.46)	↑	<0.001
Interleukin-8 (pg/mL)	4.92 (1.99)	27.57 (3.04)	↑	0.002
Soluble CD40 ligand ($x10^3$ pg/mL)	1.91 (0.18)	4.2 (0.40)	↑	0.001
Soluble P-Selectin (pg/mL)	194.55 (4.36)	206.30 (3.69)	↑	0.054
t-PA ($x10^3$ pg/mL)	5.19 (0.40)	6.39 (0.33)	-	0.377
PON-related measurements				
Lactonase activity (U/L)	6.58 (0.29)	3.19 (0.14)	↓	<0.001
Paraoxonase activity (U/L)	331.85 (13.24)	188.80 (9.28)	↓	<0.001
PON1 concentration (mg/L)	120.24 (9.65)	31.83 (1.51)	↓	<0.001
CCL2/PON1 ratio	4.35 (0.32)	26.49 (1.58)	↑	<0.001

Values are given as mean ± SEM or percentages.

However, few variables discriminated among patients according to the severity of the disease indicating dissociation between clinically obtained ABI values and laboratory measurements (Table **3**, Supportive/Supplementary Figs. **1** and **2**).

3.3. Plasma CCL2 and Serum PON1-Related Variables in Patients with PAD: Co-Localization of both Proteins in the Atherosclerotic Lesion

The correlation matrix (Supportive/Supplementary Table **4**) showed significant and positive correlations among plasma β2M, hsCRP and CCL2 levels and significant and inverse correlations with PON1 esterase and lactonase activities. Of note, PON1 concentration showed no relationship with measured enzymatic activities. These results indicate that plasma CCL2 may be considered as a potential candidate to assess inflammation in patients with clinically advanced PAD extending the concept that the design of a biomarker panel [30] may aid in clinical evaluation. All selected variables showed a high probability of predictive value as measured by odds ratio and ROC curves and such probability increased when combined (Supportive/Supplementary Fig. **3**). Plasma CCL2 concentration were consistently higher in patients (Table **2** and Fig. **1A**) than in controls and even when adjusted for confounding factors showed a significant ($p = 0.01$) predictive value for the presence of PAD (Fig. **2B**). On the other hand, serum PON1-associated activities and concentration were significantly decreased in patients when compared with controls (Table **2** and Fig. **1**).

Table 3. Laboratory Measurements in Patients with Lower Extremity Peripheral Artery Disease (PAD) and Segregated According to ABI Values

	Moderate PAD (n=54)	Severe PAD (n=31)	p-value	Adjusted OR (CI), p-value
Hemogram				
Red Blood Cells ($x10^{12}$/L)	4.26 (0.10)	4.06 (0.11)	0.240	
Hemoglobin (g/dL)	12.80 (0.31)	12.19 (0.34)	0.212	
Hematocrit (%)	38.72 (0.95)	37.08 (1.02)	0.271	
Leukocytes ($x10^9$/L)	7.72 (0.34)	8.77 (0.57)	0.096	
Platelets ($x10^9$/L)	258.39 (12.90)	259.76 (19.98)	0.952	
Fibrinogen (g/L)	6.46 (0.44)	6.18 (0.24)	0.543	
Lipid variables				
Total cholesterol, mmol/L	3.88 (0.13)	3.60 (0.14)	0.155	
HDL-cholesterol, mmol/L*	1.05 (0.47)	1.03 (0.54)	0.752	
LDL-cholesterol, mmol/L*	2.30 (0.11)	1.86 (0.15)	0.021	3.88 (0.89-16.94), 0.071
Triglycerides, mmol/L	2.82 (0.12)	2.79 (0.18)	0.898	
Glucose, mmol/L	6.46 (0.32)	6.43 (0.47)	0.953	
Insulin, pmol/L*	177.02 (39.27)	81.98 (16.48)	0.030	1.04 (1.00-1.08), 0.039
HOMA-IR*	5.79 (0.99)	2.60 (0.47)	0.005	0.61 (0.37-1.01), 0.054
Inflammation-related measurements				
β2M (mg/L)	4.06 (0.32)	4.19 (0.63)	0.854	
CCL2 (pg/mL)	664.94 (44.81)	589.16 (37.46)	0.258	
High-sensitivity CRP (mg/L)	22.51(3.30)	23.42 (5.61)	0.882	
Interleukin-6 (pg/mL)	20.76 (3.35)	23.77 (4.33)	0.585	
Interleukin-8 (pg/mL)	26.19 (6.80)	27.81 (4.52)	0.496	
Soluble CD40 ligand ($x10^3$ pg/mL)*	5.19 (0.60)	2.24 (0.38)	<0.001	1.00 (1.00-1.00), 0.018
Soluble P-Selectin (pg/mL)	207.30 (5.15)	204.10 (6.67)	0.707	
t-PA (pg/mL)	5.90 (0.77)	7.26 (0.85)	0.135	
PON-related measurements				
Lactonase activity (U/L)	3.30 (0.25)	3.25 (0.27)	0.894	
Paraoxonase activity (U/L)	192.95 (11.22)	203.70 (21.69)	0.628	
PON1 concentration (mg/L)	29.73 (1.92)	31.98 (2.14)	0.481	
CCL2/PON1 ratio	23.71 (1.72)	23.97 (2.59)	0.914	

Values are given as mean ± SEM or percentage.
*Variables included in the multivariate model. Adjusted model also included diabetes, dislipemia, HTA and their corresponding medical treatments.

Moreover, the combination of plasma CCL2 and PON1 related values, especially PON1 concentration, segregated almost perfectly controls from patients (Fig. **2A**). Although significant trends were observed, individual associations were not confirmed in the adjusted multivariate model (Fig. **2B**) but the CCL2/PON1 ratio completely predicts the presence of disease (Odds ratio= 4.3; p= 0.02; mean area under the curve= 0.995). Those patients with the highest plasma CCL2 concentration showed the lowest values of serum PON1 concentration and there were significant inverse correlations between plasma CCL2 and both paraoxonase (r= - 0.331, p < 0.001) and lactonase (r= - 0.293, p = 0.003) activities suggesting a coordinated mechanism in the pathogenesis of atherosclerosis (Supportive/Supplementary Fig. **1**). However this was not confirmed in the assessment of the degree of ICA stenosis (Supportive/Supplementary Fig. **4**). To further explore the association between CCL2 and PON1 we analyzed peripheral vascular tissues discarded in surgical procedures using immunohistochemical methods (see representative results in Fig. **3**). In most samples, the expression of CCL2 protein in adventitia was intense while PON1 staining was faint or even absent. The same trend was observed in the extracellular lipid droplets lining the

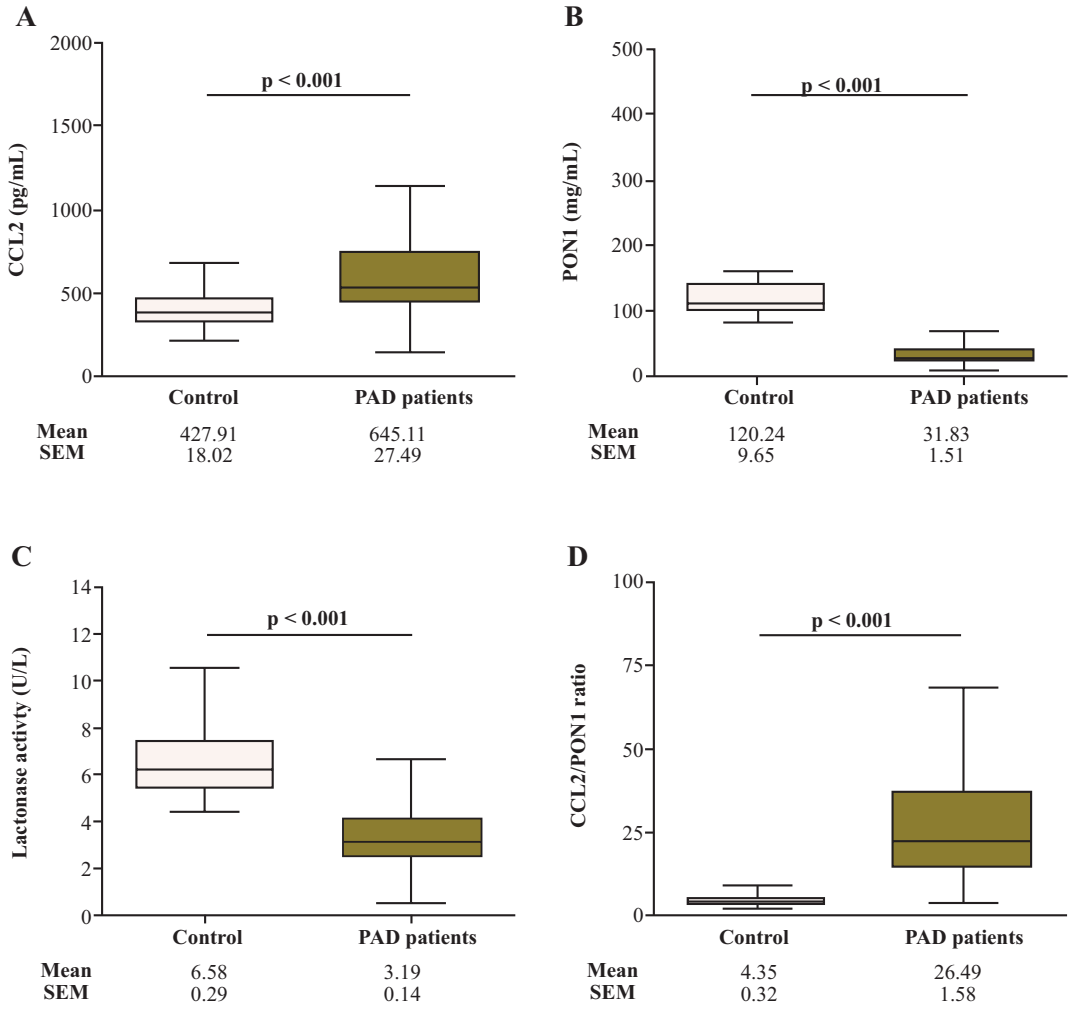


Fig. (1). Distribution of plasma CCL2 concentration (pg/mL) (**A**), serum PON1 concentration (mg/L) (**B**), lactonase activity (**C**) and CCL2/PON1 ratio (**D**) comparing patients with peripheral artery disease (PAD) and unaffected controls.

lumen. However, PON1 and CCL2 expression were co-localized in the atherosclerotic lesion. It was also evident that both proteins were increasingly expressed in more advanced lesions (not shown).

3.4. Plasma CCL2 and Serum PON1-Related Variables in Patients with CAD

We then explored this effect in relatively young patients with well documented CAD but possibly lower burden of atherosclerosis, i.e. less amount of affected arterial tissue, to test whether these changes were quantitatively different and consequently may predict the degree of endothelial damage. We found that plasma CCL2 concentration was not different in patients with CAD with respect to controls and consequently does not discriminate (Supportive/ Supplementary Fig. **5**). Also, we found that serum paraoxonase activity, although lower in CAD patients (320±10 U/L) with respect to controls was significantly higher ($p<0.001$) than that obtained in PAD patients (189±9 U/L). Serum lactonase activity in CAD patients was similar (5.85±0.14 U/L) to that obtained in controls (6.58±0.29 U/L) but significantly higher ($p<0.01$) than in PAD patients (3.19±0.14 U/L). However serum PON1 concentration was significantly different in CAD patients and there were no statistical differences with respect to that obtained in PAD patients (Table **2** and Fig. **1**) indicating that this variable may constitute an early indicator of atherosclerosis. Further, the CCL2/PON1 ratio was also clearly discriminative between CAD patients and controls. Similar genotype frequencies in the distribution of genetic variants of *CCL2* and *PON1* were found in CAD patients and controls (not shown). Taking together, these results clearly suggest that variations in CCL2 and PON1-related variables are more important in patients with PAD and probably associated with the extent of atherosclerosis.

3.5. The Distribution of Functional Genetic Variants of *CCL2* and *PON1* was not Different in Patients with PAD with Respect to that Observed in Unaffected Participants

To ascertain whether the relationship between CCL2 and PON1 was caused by a different distribution among groups of functional genetic variants, we

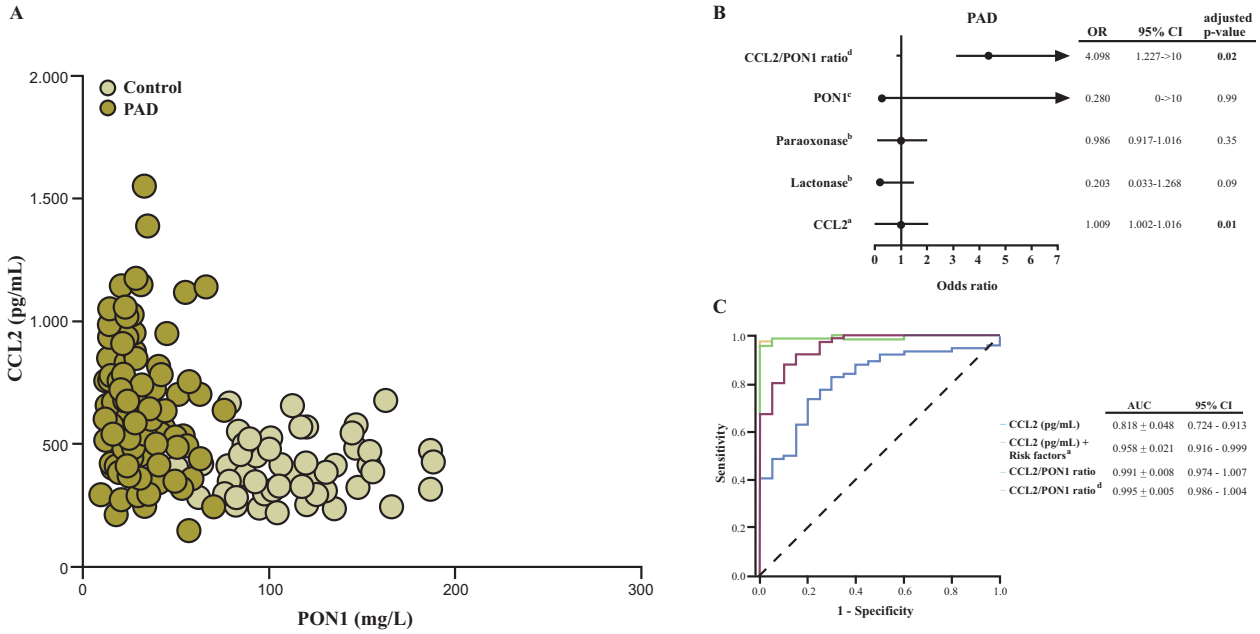


Fig. (2). Individual plasma CCL2 concentrations plotted against the corresponding PON1 concentration (**A**) and calculated relevant variables for the predicting value of proposed markers (**B**, **C**). AUC, area under the curve. Different models were constructed according to results in univariate and multivariate analysis that include [a]HOMA index, serum triglycerides concentration, plasma CCL2 concentration, BMI and smoking. [b]serum HDL-cholesterol concentration, serum paraoxonase and lactonase activities, BMI and smoking. [c]serum HDL-cholesterol concentration, BMI and smoking and [d]CCL2/PON1 ratio, HOMA index, BMI and smoking.

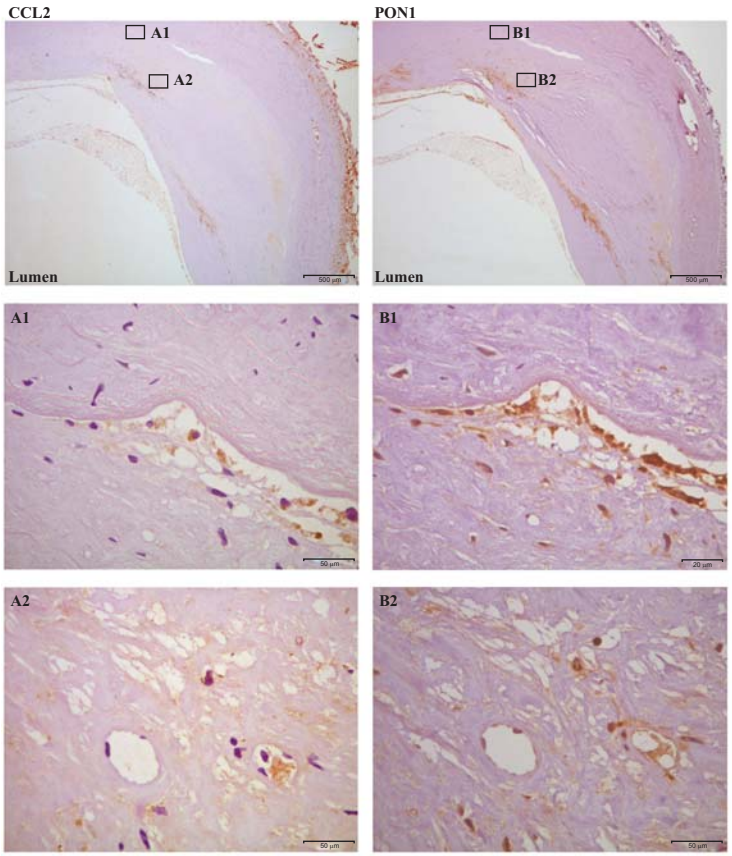


Fig. (3). Representative photomicrographs of immunohistochemical analyses of CCL2 and PON1 in a femoral arteries obtained in bypass-surgery treatment indicating a high expression of both molecules in tissue and co-localization which is more evident in regions with inflammatory cells.

genotyped the study participants for each of the indicated *CCL2* and *PON1* polymorphisms. Genotype and allelic frequencies were virtually the same in patients with respect to controls indicating that genetic effects are unlikely. The possibility of false negative results is low as no effect was detected using dominant, co-dominant and recessive models (Supportive/Supplementary Table **5** and Supportive/ Supplementary Table **6**). Further, there were also no differences when haplotypes were considered. However, linkage disequilibrium (LD) of *CCL2* polymorphisms was uniform in controls but not in patients and consequently the haplotype block used for analyses was limited to that formed with the -2835 C/A and -2578 A/G polymorphisms. LD analyses of *PON1* polymorphisms covered a unique central block region identical for patients and controls in which - 1741 C/T, + 55 L/M and + 192 Q/R polymorphisms were excluded. The expectation-maximization algorithm revealed the resulting haplotype and diplotype distribution as shown in Fig. **4**. The genotype-phenotype relationship was carefully assessed and we found that patients showed consistently higher plasma CCL2 levels and lower PON-1 concentration and activities with respect to controls in all genotypes (Supportive/Supplementary Fig. **6**). The predicted heritable linkage between -2835 C/A and -2578 A/G CCL2 genotypes was confirmed with a similar phenotype distribution (Fig. **5A**). The predicted hereditary linkage between the PON1 polymorphisms -1076 A/G and -162 G/A was only observed with respect to serum PON1 concentration. The described effect of the + 55 L/ML and + 192 Q/R polymorphisms on paraoxonase and lactonase activities was observed in both control and patients and we did not identify changes in values with respect to other genotypes (Fig. **5B** and Fig. **5C**). The relatively minor changes were independent of other clinical and laboratory variables (not shown) and were considered irrelevant to explain the inverse relationship between CCL2 and PON1-related variables.

4. DISCUSSION

According to currently accepted paradigm of the pathogenesis of atherosclerosis, factors injuring the endothelium elicit a protective, inflammatory, fibroproliferative response that becomes excessive and in its excess results in the disease process [31]. Therefore, to explore the intensity of such inflammatory response may be clinically relevant. Measurement of circulating markers of inflammation, mainly hs-CRP, has been proposed, with conflicting results, as a useful clinical procedure in order to predict adverse events in coronary arteries [32, 33]. Manifestation of atherosclerosis in lower extremity arteries, however, may show characteristics which are not seen in other clinical events. For instance, proteomic profiling and clinical studies in patients with PAD suggest that plasma β2M concentration may represent a more specific and sensitive biomarker [34]. We show that the inflammatory response is extreme in these patients, suggesting that this is causally related to advanced and extensive disease. We subsequently explored for the first time whether the inverse relationship between generally accepted mechanisms of endothelial defence against oxidative stress and the consequent inflammatory response may be detected in blood. We reasoned that patients with symptomatic PAD represent a clinical model with considerable extension to multiple diseased vessels and high burden of atherosclerosis. We found that results obtained for plasma CCL2 and serum PON1 concentrations as well as PON1 paraoxonase and lactonase activities prove our hypothesis and may constitute novel indicators of disease state that may help as biomarkers. Of note, it was also evident a clear dissociation of clinical perception, vessel permeability and inflammatory response. Our design, however, is also a limitation because in these patients it is difficult to interpret the combination of multiple variables derived from the concomitant presence of platelet dysfunction, hyperlipidemia, hypertension, diabetes, previous events and the corresponding effect of prescribed medications. The use of adjusted models, with this sample size, does not allow us to discard concealed effects but these are unlikely because the effects were universally observed in all patients. Decreased paraoxonase and lactonase activities were significantly associated with increased concentrations of circulating CCL2. However, interpretation of plasma CCL2 is difficult. Although in other conditions plasma CCL2 concentration was independent of other inflammatory variables [35, 36] we have shown a good correlation with plasma β2M and hsCRP levels, partially confirming the in vitro effects of certain circulating molecules on chemokine expression in human endothelial cells [37-39]. Moreover, the influence of endothelial receptors on the concentration of circulating chemokines has been recently described and we have previously shown that measurements in serum and plasma may be significantly different and dependent on certain genetic variants [40]. We therefore propose the use in combination of both the higher CCL2 associated with lower PON1, especially the CCL2 to PON1 ratio, which clearly differentiated patients with respect to controls and showed a strong, nearly complete, predictive value. We acknowledge that our clinical approach cannot demonstrate a coordinated role between both molecules but suggest a strong systemic response. The issue is further complicated by the fact that our previous studies in vitro and in animal models pioneeringly revealed apparently contradictory mechanisms [13, 15]. At the cellular level [13] PON1 seems to modulate CCL2 production, but both are ubiquitously distributed in most tissues [15]. Moreover, in diseased vessels, expression of both proteins is increased indicating that observations in plasma do not necessary correlate with the actual role at the molecular level. This is obviously irrelevant for diagnostic purposes but suggests the results may have a prognostic value that should be further ascertained in future prospective studies, mainly in asymptomatic patients. Our results in patients with CAD, with possibly

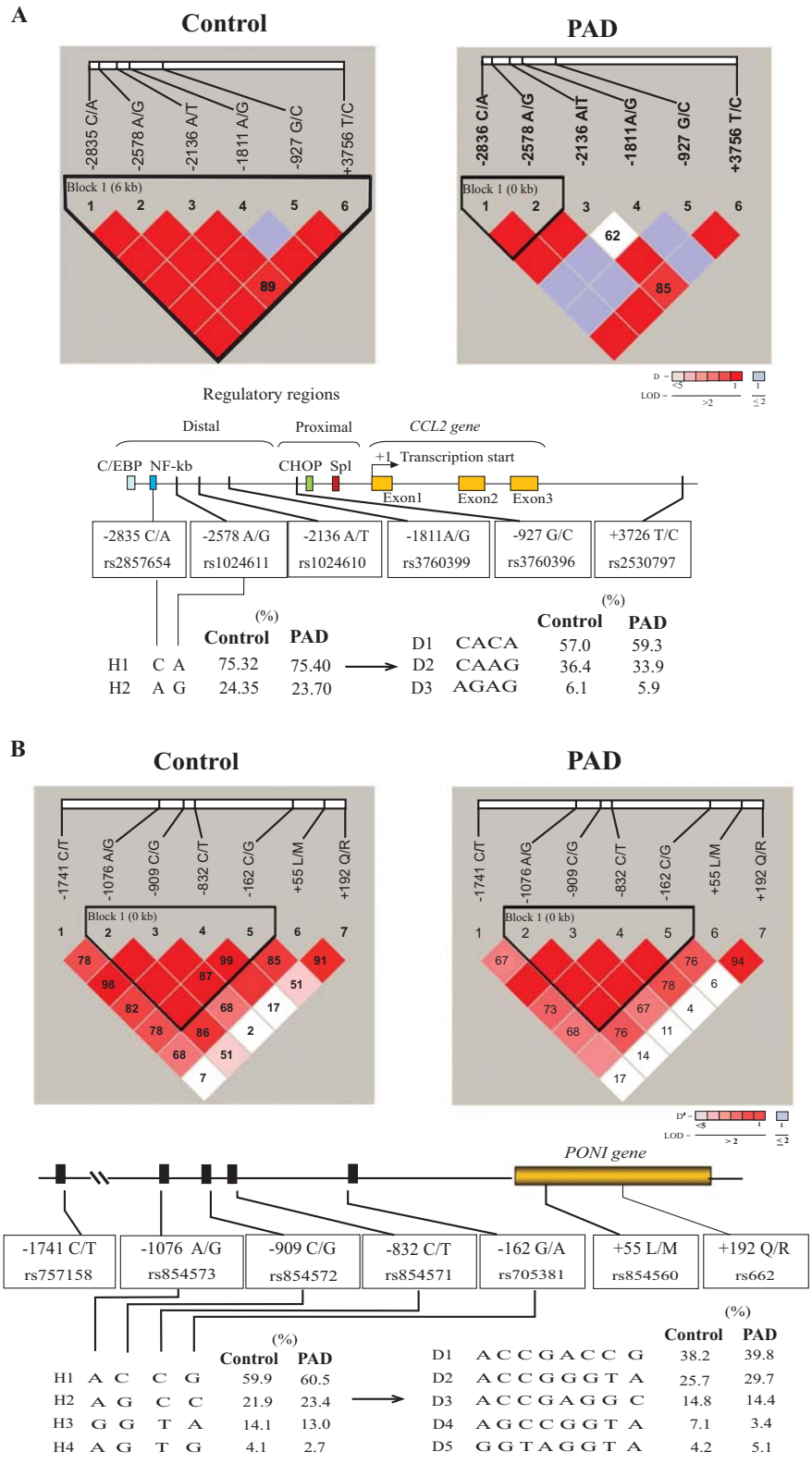


Fig. (4). Linkage disequilibrium (LD) structures across *CCL2* (**A**) and *PON1* (**B**) calculated in Haploview software and labelled by their gene position. Pairwise linkage disequilibrium (D′) values are given in each square intersecting for each pair of SNPs and haplotype blocks outlined. The darker the square, the greater the linkage disequilibrium between the SNPs (Blank square indicates D'=1.0). Gene schematic position of the 6 *CCL2* (**A**) and the 7 *PON1* (**B**) genotyped SNPs, and haplotypes and diplotypes estimated using the expectation-maximisation algorithm. For each SNP, it is indicated their unique RS number and the name referred to their gene position.

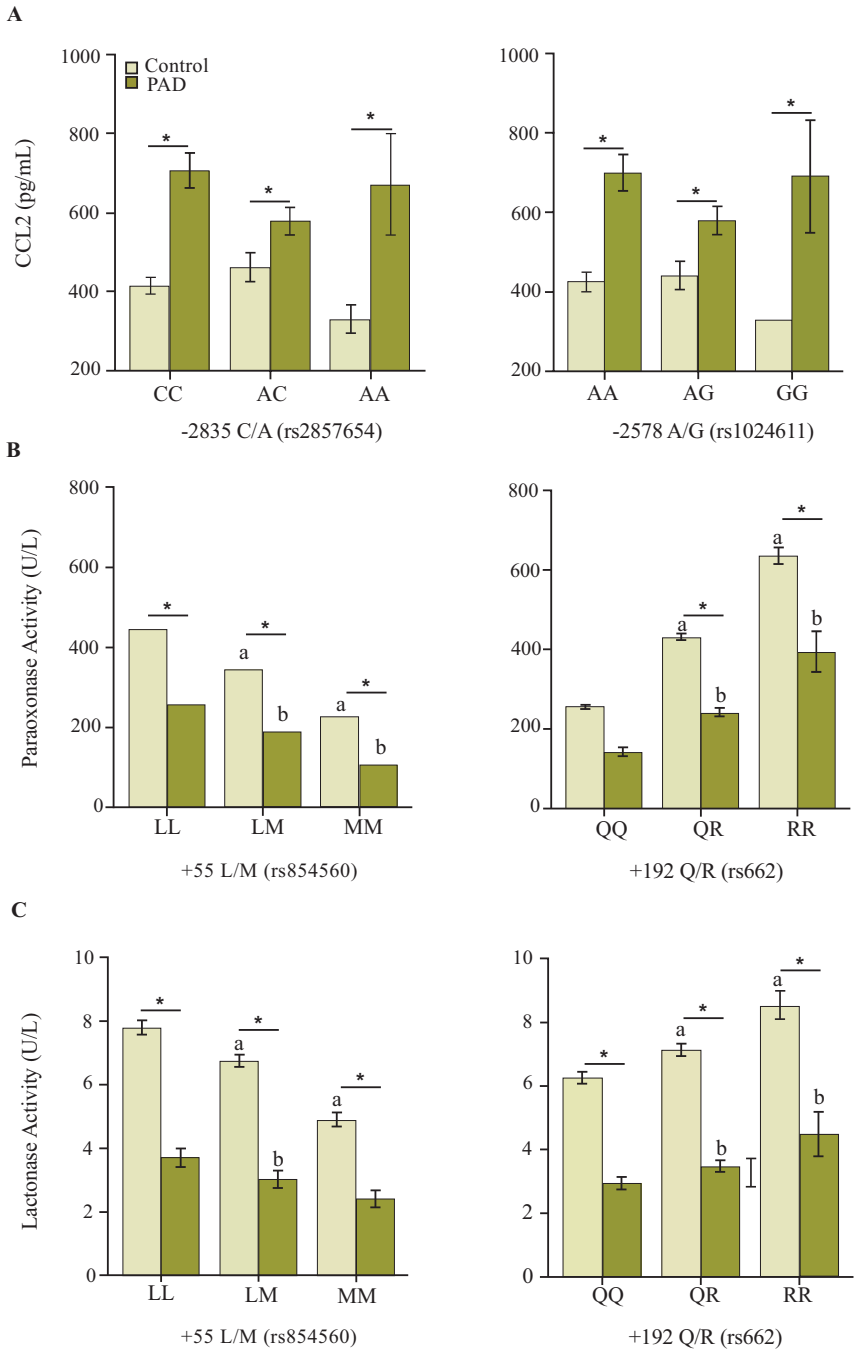


Fig. (5). Comparisons of plasma CCL2 concentration according to the CCL2 genotype variants -2835 C/A and -2578A/G (**A**) and the effect of +55 L/M and +192 Q/R *PON1* genotype variants in serum paraoxonase activity (**B**) and lactonase activity (**C**). *p<0.05 in PAD patients compared to controls. [a]p<0.05 Difference in genetic variants compared to the most common genotype in each SNPs from control group. [b]p<0.05 Difference in genetic variants compared to the most common genotype in each SNPs from PAD patients.

a lower burden of atherosclerosis are also suggestive of a coordinated role of PON1 and CCL2 and possibly of a quantitative relationship. Contrary to findings in patients with PAD, we did not find significant changes in plasma CCL2 or PON1 lactonase with respect to controls and only slightly lower values of PON1 paraoxonase activity but circulating PON1 concentration was lower indicating a possible predictive value of the CCL2 to PON1 ratio on the extent of atherosclerosis. Comparison between both groups also highlight that decrease in PON1 activity is mainly dependent on the extension of the disease rather than on decreased HDL-cholesterol levels.

We have also shown that the effects of methodological constraints [41] are unlikely, and are not the consequence of associated genetic factors because genotype and allelic frequencies were similar between controls and patients and this was observed in

both cohorts. Minor differences in haplotype and diplotype distribution, according to our previous data [26, 27] were considered tangential to this issue although the effect of other genetic factors cannot be discarded [42]. Of note, future research should also include the effect of decreased PON1 on the effectiveness of commonly prescribed anti-platelet therapy in these patients because PON1 seems to be a determinant of metabolism and biotransformation of both aspirin and clopidogrel [21, 22].

Finally, we have found in a previous report in human aortas and now in human peripheral arteries that immunostaining of PON1 and CCL2 increase with the progression of atherosclerosis, possibly as a response to both increased cellular oxidative stress and the migration of monocytes [25, 43]. It is therefore evident that the decrease in circulating PON1 related variables may indicate an independent mechanism representing a systemic failure to provide antioxidant elements that may increase the inflammatory response.

5. CONCLUSION

In conclusion, our results identify clinically an inverse relationship between CCL2 and PON1 that is independent of genetic variations and that the relationship between defense against oxidative stress and inflammation may be detected using simple laboratory measurements in patients with atherosclerosis. Quantitative differences may also represent an indicator of the extent of atherosclerosis.

ACKNOWLEDGEMENTS

This work was supported by grant PI08/1381 from the *Instituto de Salud Carlos III* (ISCIII, Madrid, Spain), and the Fondo Europeo de desarrollo regional (FEDER). Anna Rull is the recipient of a fellowship from the Generalitat de Catalunya (FI-G 0503).

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publishers Web site along with the published article.

REFERENCES

[1] Criqui MH. Peripheral arterial disease--epidemiological aspects. Vasc Med 2001; 6: 3-7.
[2] Mehlsen J, Wiinberg N, Joergensen BS, Schultz-Larsen P. High prevalence of peripheral arterial disease in patients with previous cerebrovascular or coronary event. Blood Press 2010; 19: 308-12.
[3] McDermott MM, Liu K, Greenland P, *et al*. Functional decline in peripheral arterial disease: associations with the ankle brachial index and leg symptoms. JAMA 2004; 292: 453-61.
[4] Gornik HL, Creager MA. Contemporary management of peripheral arterial disease: Cardiovascular risk-factor modification. Cleve Clin J Med 2006; 73 Suppl4: S30-37.
[5] Hirsch AT, Criqui MH, Treat-Jacobson D, *et al*. Peripheral arterial disease detection, awareness, and treatment in primary care. JAMA 2001; 286: 1317-24.
[6] Criqui MH, Langer RD, Fronek A, *et al*. Mortality over a period of 10 years in patients with peripheral arterial disease. N Engl J Med 1992; 326: 381-6
[7] Murabito JM, Keyes MJ, Guo CY, *et al*. Cross-sectional relations of multiple inflammatory biomarkers to peripheral arterial disease: The Framingham Offspring Study. Atherosclerosis 2009; 203: 509-14.
[8] Cooke JP, Wilson AM. Biomarkers of peripheral arterial disease. J Am Coll Cardiol 2010; 55: 2017-23.
[9] McDermott MM, Lloyd-Jones DM. The role of biomarkers and genetics in peripheral arterial disease. J Am Coll Cardiol 2009; 54: 1228-37.
[10] Cesari M, Penninx BW, Newman AB, *et al*. Inflammatory markers and onset of cardiovascular events: results from the Health ABC study. Circulation 2003; 108: 2317-22.
[11] Feldmann M. Many cytokines are very useful therapeutic targets in disease. J Clin Invest 2008; 118: 3533-6.
[12] Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. Circ Res 2004; 95: 858-66.
[13] Mackness B, Hine D, Liu Y, Mastorikou M, Mackness M. Paraoxonase-1 inhibits oxidised LDL-induced MCP-1 production by endothelial cells. Biochem Biophys Res Commun. 2004; 318: 680-3.
[14] Rodríguez-Sanabria F, Rull A, Beltrán-Debón R, *et al*. Tissue distribution and expression of paraoxonases and chemokines in mouse: The ubiquitous and joint localisations suggest a systemic and coordinated role. J Mol Histol 2010; 41: 379-86.
[15] Rull A, Camps J, Alonso-Villaverde C, Joven J. Insulin resistance, inflammation, and obesity: role of monocyte chemoattractant protein-1 (or CCL2) in the regulation of metabolism. Mediators Inflamm 2010; 2010. pii: 326580.
[16] Petrkova J, Szotkowska J, Hermanova Z, Lukl J, Petrek M. Monocyte chemoattractant protein-1 in patients with peripheral arterial disease. Mediators Inflamm 2004; 13: 39-43.
[17] van Wijk DF, van Leuven SI, Sandhu MS, *et al.* Chemokine Ligand 2 Genetic Variants, Serum Monocyte Chemoattractant Protein-1 Levels, and the Risk of Coronary Artery Disease. Arterioscler Thromb Biol 2010; 30: 1460-6.
[18] Marsillach J, Aragonès G, Beltrán R, *et al.* The measurement of the lactonase activity of paraoxonase-1 in the clinical evaluation of patients with chronic liver impairment. Clin Biochem 2009; 42: 91-8.
[19] Ferré N, Marsillach J, Camps J, *et al*. Paraoxonase-1 is associated with oxidative stress, fibrosis and FAS expression in chronic liver diseases. J Hepatol 2006; 45: 51-9.
[20] Pasqualini L, Cortese C, Marchesi S*, et al*. Paraoxonase-1 activity modulates endothelial function in patients with peripheral arterial disease. Atherosclerosis 2005; 183: 349-54.
[21] Camps J, Marsillach J, Joven J. The paraoxonases: role in human diseases and methodological difficulties in measurement. Crit Rev Clin Lab Sci 2009; 46: 83-106.
[22] Bouman HJ, Schömig E, van Werkum JW, *et al.* Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 2011; 17: 110-6.
[23] Yun WS, Rho YN, Park UJ, Lee KB, Kim DI, Kim YW. Prevalence of asymptomatic critical carotid artery stenosis in Korean patients with chronic atherosclerotic lower extremity ischemia: is a screening carotid duplex ultrasonography worthwhile? J Korean Med Sci 2010; 25: 1167-70.
[24] Fowkes FG, Murray GD, Butcher I, *et al*. Ankle brachial index combined with Framingham Risk Score to predict cardiovascular events and mortality: a meta-analysis. JAMA 2008; 300: 197-208.
[25] Marsillach J, Camps J, Beltran-Debón R, *et al*. Immunohistochemical analysis of paraoxonases-1 and 3 in human atheromatous plaques. Eur J Clin Invest 2011; 41: 308-14.
[26] Alonso-Villaverde C, Aragonés G, Beltrán-Debón R, *et al*. Host-Pathogen Interactions in the Development of Metabolic Disturbances and Atherosclerosis in HIV Infection: The Role of CCL2 Genetic Variants. Cytokine 2010; 51: 251-8.

[27] Parra S, Marsillach J, Aragonés G, *et al*. Paraoxonase-1 gene haplotypes are associated with metabolic disturbances, atherosclerosis, and immunologic outcome in HIV-infected patients. J Infect Dis 2010; 201: 627-3.
[28] Dean AG, Sullivan KM, Soe MM. OpenEpi: Open Source Epidemiologic Statistics for Public Health, Version 2.3.1. www.OpenEpi.com
[29] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005; 21: 263-5.
[30] Fung ET, Wilson AM, Zhang F, *et al*. A biomarker panel for peripheral arterial disease. Vasc Med 2008; 13: 217-24.
[31] Ross R. Cell biology of atherosclerosis. Annu Rev Physiol 1995; 57: 791-804.
[32] Danesh J, Wheeler JG, Hirschfield GM, *et al*. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med 2004; 350: 1387-97.
[33] Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med 2000; 342: 836-43
[34] Wilson AM, Kimura E, Harada RK, *et al*. Beta2-microglobulin as a biomarker in peripheral arterial disease: proteomic profiling and clinical studies. Circulation 2007; 116: 1396-4103.
[35] Alonso-Villaverde C, Coll B, Parra S, *et al*. Atherosclerosis in patients infected with HIV is influenced by a mutant monocyte chemoattractant protein-1 allele. Circulation. 2004; 110: 2204-9.
[36] Hoogeveen RC, Morrison A, Boerwinkle E, *et al*. Plasma MCP-1 levels and risk for peripheral arterial disease and incident coronary Heart disease: Atherosclerosis Risk in Communities study. Atherosclerosis 2005; 183: 301-7.
[37] Garlichs CD, John S, Schmeisser A, *et al*. Upregulation of CD40 and CD40 ligand (CD154) in patients with moderate hypercholesterolemia. Circulation 2001; 104: 2395-400.
[38] Devaraj S, Kumaresan PR, Jialal I. Effect of C-reactive protein on chemokine expression in human aortic endothelial cells. J Mol Cell Cardiol 2004; 36: 405-10.
[39] Pasceri V, Cheng JS, Willerson JT, Yeh ET. Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. Circulation 2001; 103: 2531-4.
[40] Schnabel RB, Baumert J, Barbalic M, *et al*. Duffy antigen receptor for chemokines (Darc) polymorphism regulates circulating concentrations of monocyte chemoattractant protein-1 and other inflammatory mediators. Blood. 2010; 115: 5289-99.
[41] Parra S, Marsillach J, Aragonés G, *et al*. Methodological constrains in interpreting serum paraoxonase-1 activity measurements: an example from a study in HIV-infected patients. Lipids Health Dis 2010; 9: 32.
[42] Knowles JW, Assimes TL, Li J, Quetermous T, Cooke JP. Genetic susceptibility to peripherial arterial disease: A dark corner in vascular biology. Arterioscler Thromb Vasc Biol 2007; 27: 2068-78.
[43] Mackness B, Hunt R, Durrington PN, Mackness MI. Increased immunolocalization of paraoxonase, clusterin, and apolipoprotein A-I in the human artery wall with the progression of atherosclerosis. Arterioscler Thromb Vasc Biol 1997; 17: 1233-8.

Received: March 01, 2011 Revised: April 06, 2011 Accepted: April 18, 2011

ESTUDIO 2

Serum paraoxonase-3 concentration is associated with insulin sensitivity in peripheral artery disease and with inflammation in coronary artery disease

Atherosclerosis 2012, 220(2): 545-51

En el presente estudio se describe, por primera vez, las alteraciones en las concentraciones séricas de PON3 en pacientes con coronariopatía(CAD) y con aterosclerosis periférica (EAP).

Nuestros pacientes con enfermedad coronaria o arterial periférica tenían un aumento significativo de las concentraciones de PON3 en suero, que fue acompañado por una disminución de las concentraciones de PON1 (y actividades en EAP) y la disminución de niveles de colesterol HDL. Esto implica un enriquecimiento relativo de las partículas de HDL en PON3 en estos pacientes, que es, posiblemente, un mecanismo compensatorio para contrarrestar la disminución de PON1. Esta conclusión está de acuerdo con los estudios experimentales que muestran que la sobreexpresión de PON3 humana en ratones protege a estos animales de la progresión de la aterosclerosis.

Un hallazgo interesante del presente artículo es la relación observada entre las concentraciones séricas PON3, hipertensión arterial y alteraciones metabólicas en la EAP, y en particular con la insulina y el índice HOMA (Homeostasis Model Assessment), que no se ha encontrado en pacientes con CAD.

La diabetes es la plataforma de la enfermedad subyacente más común que causa EAP, y estos resultados sugieren un papel de PON3 en la regulación del metabolismo de la glucosa, que es uno de los trastornos más importantes relacionados con el síndrome metabólico. En la EAP, sin embargo, las concentraciones séricas de PON3 no fueron asociadas a marcadores inflamatorios circulantes como la β2M, MCP-1 o PCR, una asociación que se observó de manera positiva en el CAD. No sabemos la razón de estas diferencias, pero las concentraciones séricas de PON3 fueron significativamente menores en la EAP, mientras que los niveles de marcadores inflamatorios fueron mucho más altos. Existe la posibilidad de que la magnitud de la extensión física de las áreas afectadas en la EAP hace que la PON3 no pueda impedir la inflamación masiva arterial en estos pacientes.

Atherosclerosis 220 (2012) 545–551

Contents lists available at SciVerse ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Serum paraoxonase-3 concentration is associated with insulin sensitivity in peripheral artery disease and with inflammation in coronary artery disease

Anna Rull[a,1], Raúl García[b,1], Laura Fernández-Sender[a], Anabel García-Heredia[a], Gerard Aragonès[a], Raúl Beltrán-Debón[a], Judit Marsillach[c], Josep M. Alegret[d], Vicente Martín-Paredero[b], Bharti Mackness[a], Michael Mackness[a], Jorge Joven[a], Jordi Camps[a,*]

[a] *Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, C. Sant Joan s/n, 43201 Reus, Catalonia, Spain*
[b] *Servei d'Angiologia, Cirurgia Vascular i Endovascular, Hospital Universitari Joan XXIII, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Tarragona, Catalonia, Spain*
[c] *Department of Genome Sciences, University of Washington, Seattle, WA, USA*
[d] *Unitat de Cardiologia, Hospital Universitari de Sant Joan, Institut d'Investigació Sanitària Pere Virgili, Universitat Rovira i Virgili, Reus, Catalonia, Spain*

ARTICLE INFO

Article history:
Received 6 July 2011
Received in revised form 14 October 2011
Accepted 14 November 2011
Available online 22 November 2011

Keywords:
Antioxidants
Atherosclerosis
Lipoproteins
Myocardial infarction
Paraoxonases
Peripheral vascular disease

ABSTRACT

Objective: There are no data on the relationship between serum paraoxonase-3 (PON3) concentration and atherosclerosis in humans. Our aim was to investigate possible associations, using recently developed methods, in patients with peripheral artery disease (PAD) or coronary artery disease (CAD).
Methods: We studied 118 PAD and 72 CAD patients and 175 healthy volunteers. Serum PON3 was determined by in-house ELISA using polyclonal antibodies generated against a synthetic peptide with a sequence specific to PON3. Polymorphisms of the *PON3* promoter were analysed by the Iplex Gold MassArray™ method.
Results: There was a significant increase in serum PON3 concentration in both groups of patients with respect to the control group. In PAD patients, we observed significant positive correlations between PON3, insulin levels and HOMA index. These associations were not observed in CAD. There were significant positive associations between serum PON3 and β-2-microglobulin, CCL2 and high-sensitivity C-reactive protein in CAD patients, but not in PAD. We did not find any significant differences in *PON3* gene promoter polymorphisms and their haplotypes between patients and controls, indicating that associations were not genetically determined.
Conclusion: In both atherosclerotic phenotypes, serum PON3 concentration was increased, but this was associated with decreased insulin sensitivity in PAD and with inflammation in CAD.

1. Introduction

Atherosclerosis remains one of the leading causes of morbidity and mortality worldwide. The pathobiology of this disease is characterised by a sustained and silent progression, and the clinical consequences may vary depending on the anatomic localisation of the lesions. Patients with coronary artery disease (CAD) may develop sudden death or myocardial infarction, sometimes at relatively young age. On the other hand, patients with peripheral artery disease (PAD), have a higher burden of atherosclerosis than those patients with adverse events in other arterial territories, and this represents a differential aspect, since they can develop advanced and extensive arterial alterations without any immediate threat to survival. However, both forms of atherosclerosis share some underlying molecular mechanisms. To-date, it is generally accepted that one of the main metabolic causes of atherosclerosis is oxidative stress and the associated increased lipid peroxidation, leading to the activation of inflammatory cells. Thus, the enzymes involved in protection against oxidative stress have received a great deal of attention in recent years [1].

Paraoxonases (PON) are a family of enzymes comprising 3 members, *PON1*, *PON2* and *PON3*, whose genes are located adjacent to each other on chromosome 7q21-22 [2]. In humans, PON1 and PON3 genes are produced in many cell types [3], and their protein products are found in the circulation bound to high-density lipoproteins (HDL) [4]. Conversely, PON2 is an intracellular enzyme which is not, however, found in plasma [5]. All these enzymes are able to retard low-density lipoprotein (LDL) oxidation and cellular

* Corresponding author. Tel.: +34 977 310300; fax: +34 977 312569.
E-mail addresses: jcamps@grupsagessa.com, jcamps@grupsagessa.cat (J. Camps).
[1] These authors contributed equally to this work.

0021-9150/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2011.11.021

oxidative stress [6]. In addition, studies using a variety of mouse models of atherosclerosis have consistently shown that human PON1, 2 or 3 expression inhibits, or reverses, the development of atherosclerosis via mechanisms involving the reduction of oxidative stress, the promotion of cholesterol efflux from macrophages, and the normalisation of vascular endothelium function [6].

Although the knowledge on PON1 and PON2 structure and function is rapidly expanding, not much is known about the PON3 protein. Its gene was identified in 1996 when Primo-Parmo et al. [2] detected a large number of cDNA sequences in the Genome Data Base similar to human PON1. The percentage identity among human *PON1*, *PON2*, and *PON3* genes is high (about 70%) and the genes are believed to derive from a common precursor [7]. Clinical research on PON3 has been hampered by the lack of methods for measurement [5], but we recently described a high-throughput, reliable enzyme-linked immunosorbent assay (ELISA) to analyse PON3 concentration in human serum [8]. The present study was aimed to investigate the possible association between serum PON3 concentration with PAD and CAD.

2. Methods

2.1. Study population

Patients with clinically diagnosed symptomatic PAD ($n = 118$, 85.6% men, 55–80 years old) were recruited from the outpatient clinics of *Hospital Universitari Joan XXIII*. At entrance, relevant data were collated by clinical records, interview and physical examination. Selected laboratory parameters were measured as well as the ankle to brachial index (ABI) and the degree of internal carotid artery (ICA) stenosis using widely accepted methods and recommendations [9,10]. The ABI testing was performed with the patient in the supine position, after 5 min of rest, using a standard protocol. The brachial blood pressure measurement was obtained in the left arm, and in the right and left dorsalis pedis arteries, using a 5–7 mHz hand-held Doppler. The ABI was calculated by dividing the ankle blood pressure by the brachial blood pressure for each lower extremity. Chronic ischemia symptoms were detected using the Fontaine classification, a standardised physician-administered questionnaire that inquires about the presence of exertional calf discomfort related to walking uphill or walking rapidly. The degree of ICA was classified using the duplex criteria developed by the University of Washington based on the percentage of arterial diameter reduction (Supplementary Table 1). They had no signs of infection, renal impairment, liver disease, neoplasia, or autoimmune diseases.

Subjects with CAD ($n = 72$, all men, 47–51 years old) were patients that survived to an episode of myocardial infarction and were recruited in the Cardiology Unit of the *Hospital Universitari de Sant Joan*. They were matched with PAD patients for body mass index (BMI), incidence of diabetes, hypertension and corresponding treatments. The proportion of smokers and non-smokers was also similar in both groups. In these patients, there were no significant alterations in ICA, and ABI measurements were >1.0.

The control group consisted of 175 healthy volunteers (42.9% men, 50–80 years old) participating in an epidemiological study as described previously [11]. We recruited those volunteers that, after an interview, attended a clinical examination and provided a fasting blood sample. In the control group overweight was common (28.96 ± 0.30 kg/m^2) and current smoking habit was less than 50% (23.6%). Incidence of diabetes, defined by history, use of diabetes medication or fasting serum glucose levels ≥6.99 mmol/L, was present in an 8% of subjects. Dyslipidaemia, considered to be present by previous diagnosis, medication use or high levels of LDL-cholesterol (≥4.13 mmol/L) and/or low levels of HDL-cholesterol (≤1.03 mmol/L), and hypertension, defined by history and medication use, were not present in the control group. Also, there was no clinical analytical evidence of renal insufficiency, major liver disease, neoplasia or neurological disorders in these participants.

A fasting venous sample blood was obtained from all the participants and serum and EDTA-plasma were stored at −80 °C until measurements were performed. 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 Committees of the participating hospitals (Institutional Review Boards).

2.2. Measurement of serum PON1 and PON3 levels

Serum PON1 and PON3 concentrations were determined by in-house ELISA with rabbit polyclonal antibodies generated against synthetic peptides with sequences specific of mature PONs. The employed peptides were CRNHQSSYQTRLNALREVQ (specific for PON1) and CRVNASQEVEPVEPEN (specific for PON3). Details of these methods have been previously reported [8,12]. Serum PON1 lactonase activity was analysed by measuring the hydrolysis of 5-thiobutyl butyrolactone (TBBL) as described [13,14]. Lactonase activity was measured in an assay reagent containing 1 mM

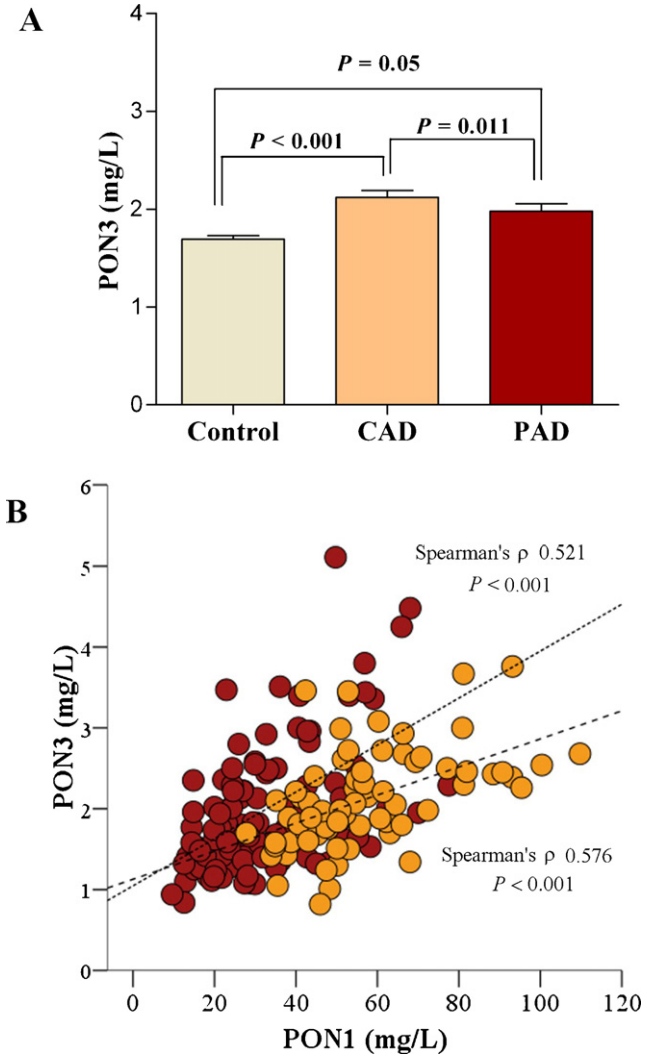


Fig. 1. (A) Serum PON3 concentrations in control subjects and patients with coronary artery disease (CAD) and peripheral artery disease (PAD). (B) Relationships between serum PON1 and PON3 concentrations in patients with CAD (yellow dots) and PAD (red dots). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

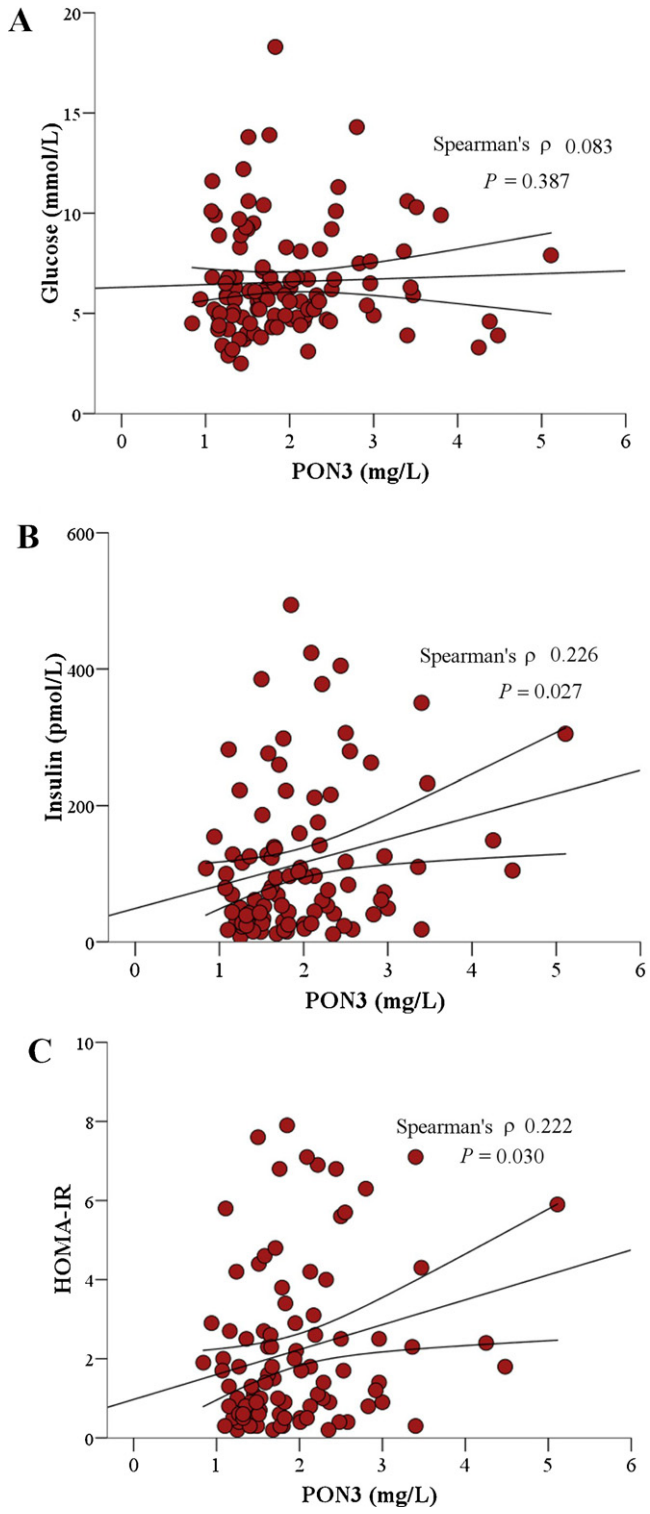


Fig. 2. Relationships between serum PON3 concentrations and serum glucose, insulin and the HOMA index in patients with peripheral artery disease (PAD).

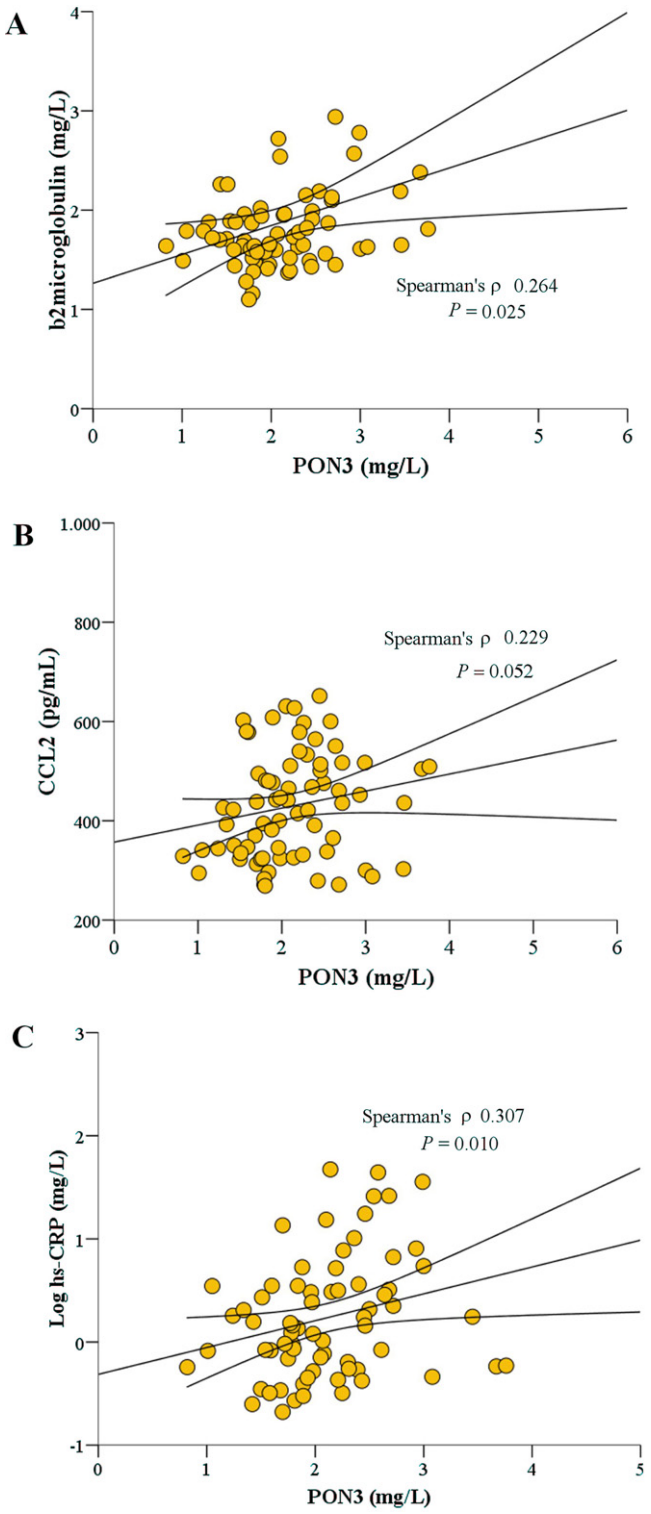


Fig. 3. Relationships between serum PON3 concentrations and inflammatory markers in patients with coronary artery disease (CAD).

$CaCl_2$, 0.25 mM TBBL and 0.5 mM 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) in 0.05 mM Tris–HCl buffer, pH 8.0. The change in absorbance was monitored at 412 nm. Activities were expressed as U/L (1U = 1 mmol of TBBL hydrolysed per minute). Serum PON1 esterase activity was determined by measuring the rate of hydrolysis of paraoxon at 410 nm and 37 °C in a 0.05 mM glycine buffer, pH 10.5 with 1 mM $CaCl_2$ [15]. Activities were expressed as U/L (1U = 1 μmol of paraoxon hydrolysed per minute).

2.3. Other biochemical measurements

Serum β-2-microglobulin (β2M), high-sensitivity C reactive protein (hs-CRP), glucose, insulin, cholesterol, triglycerides, and HDL-cholesterol concentrations were measured in an automated analyser (UniCel™ DxI 800, Beckman Coulter, Fullerton, CA, USA). LDL-cholesterol concentrations were estimated by the Friedewald formula [16]. Insulin resistance was estimated by using the

homeostasis model assessment index (HOMA-IR) [17]. Plasma CCL2 concentration was measured by flow cytometry (FlowCytomix, Bender MedSystems).

2.4. PON3 genotyping

Genomic DNA was obtained from leukocytes (Puregene DNA Isolation reagent set, Gentra Systems Inc., Minneapolis, MN, USA). Selected single nucleotide polymorphisms (SNP's) of the *PON3* promoter were analysed by the Iplex Gold MassArray™ method (Sequenom Inc., San Diego, CA, USA) at the Spanish National Genotyping Center (*Centro Nacional de Genotipado*, *Universitat Pompeu Fabra*, Barcelona, Spain).

2.5. Statistical analysis

Sample size was calculated with Open Epi (Open Epi: Open Source Epidemiologic Statistics for Public Health, Version 2.3.1. www.Openepi.com) using data from previously accepted biomarkers and own results [18]. The normality of distributions was determined with the Kolmogorov–Smirnov test. Differences between two groups were assessed with the Student's *t*-test (parametric) or the Mann–Whitney *U*-test (non-parametric). Differences between multiple groups were analysed by the Kruskal–Wallis test. Pearson or Spearman correlation coefficients were used to evaluate the degree of association between variables. Each SNP was tested for Hardy–Weinberg equilibrium using Haploview 4.0 software [19]. Estimates of linkage disequilibrium between SNPs were calculated using Fisher's test. Results are shown as means and SEM in parenthesis. The SPSS 18.0 package was employed for all statistical calculations.

3. Results

3.1. Serum PON3 and PON1 levels and other biochemical variables in CAD and PAD patients

Results of the biochemical measurements in patients with CAD or PAD compared to those obtained in the control group are shown in Fig. 1 and Table 1. There was a significant increase in serum PON3 concentration in the CAD and PAD patients with respect to the control group. Both groups of patients showed significantly lower levels of HDL-cholesterol, LDL-cholesterol and higher serum triglyceride concentrations. All PON1-related variables (serum PON1 concentration, PON1 lactonase and paraoxonase activities) were significantly lower in PAD patients, but not in CAD patients who only presented a significant decrease in serum PON1 concentration compared to the control group. β2M and hs-CRP concentrations were higher in CAD and PAD patients than in normal subjects. CCL2 concentration was only significantly higher in PAD patients. It was also evident from Table 1 that the lowest values of PON1 activity were found in PAD with little overlapping with CAD and controls.

We observed significant positive associations between serum PON3 and PON1 concentrations in both CAD and PAD patients (Fig. 1B), which were not observed in the control group ($\rho = 004$, $P = 0.958$). No significant associations between serum PON3 concentrations and PON1 lactonase and paraoxonase activities was observed in any of the groups (Supplementary Table 2).

When PAD patients were characterised by Fontaine classification, no significant differences were observed between groups in relation to biochemical parameters, inflammatory markers or PON-related measurements, although PON1 and PON3 concentrations showed a trend to be higher (Supplementary Fig. 1 and Table 3).

3.2. Relationships between serum PON3 concentrations and the clinical characteristics of the patients

No association was found between serum PON3 concentration and gender, smoking status or diabetes diagnosis in PAD and CAD patients. Only in PAD patients, we observed a significant positive correlation between PON3 and insulin levels and HOMA index (Fig. 2). These associations were not observed in CAD [Insulin: $\rho = 0.017$; $P = 0.887$; HOMA index: $\rho = 0.026$; $P = 0.828$]. Also in PAD patients we observed a significant positive association between serum PON3 concentrations and the presence of hypertension [2.07 (0.11) vs 1.67 (0.08) mg/L; $P = 0.038$], not observed in CAD patients. When the influence of antihypertensive treatments in PAD patients was evaluated, a significant positive relationship was observed between PON3 and the combination of angiotensin converting enzyme inhibitor (ARE) and angiotensin receptor blocker (ARB) treatment [2.21 (0.13) vs 1.82 (0.08) mg/L; $P = 0.019$). Multiple regression analysis confirmed the significant positive association between serum PON3 concentration and insulin in PAD patients (Supplementary Table 4).

3.3. Relationships between serum PON3 concentrations and inflammation-related markers

There were significant positive associations between serum PON3 levels and β2M, CCL2 and hs-CRP concentrations in CAD patients (Fig. 3). However, these associations were not observed in subjects with PAD (Supplementary Fig. 2). Most PAD patients had elevated serum CRP concentrations, indicating an acute inflammatory condition. Multiple regression analysis confirmed the significant positive association between serum PON3 concentration and β2M in CAD patients (Supplementary Table 5).

As described in Section 2, all PAD patients were symptomatic, and most were included in grades III and IV of the Fontaine classification, meaning that most of them had ulceration or gangrene lesions. Less than 5% of PAD patients had serum CRP concentration $\leq$5 mg/L. These patients had similar serum PON3 concentration than the control subjects (Supplementary Fig. 3).

3.4. Influence of genotypic variants on serum PON3 concentrations in CAD and PAD patients

The distribution of *PON3* genotype variants among cases and controls and the Odds Ratios (95% CI) for CAD and PAD risk in relation to *PON3* genetic polymorphisms are shown in Supplementary Tables 6 and 7. More than 95% of the subjects were successfully typed in each *PON3* polymorphism. Genotype frequencies of each *PON3* polymorphism did not deviate from the Hardy–Weinberg equilibrium, neither in patients nor in controls. Linkage disequilibrium analyses revealed that all six *PON3* polymorphisms covered a unique central block region identical for patients and controls (Fig. 4A). The expectation-maximization algorithm revealed three possible combinations with similar percentage frequency in all of the groups, but the frequency of ATGTGT showed a trend to be minor in control subjects than in patients.

Finally, the genotype–phenotype relationship was carefully assessed in control and patients. We observed that $PON3_{-746}$, $PON3_{-665}$ and $PON3_{-567}$ polymorphisms were those more significantly associated with changes in PON3 concentrations in the different groups (Fig. 4B).

4. Discussion

Oxidative stress and inflammation play a key role in atherosclerosis [20–22]. Atherosclerotic plaque formation and progression is characterised by endothelial cell dysfunction, accumulation

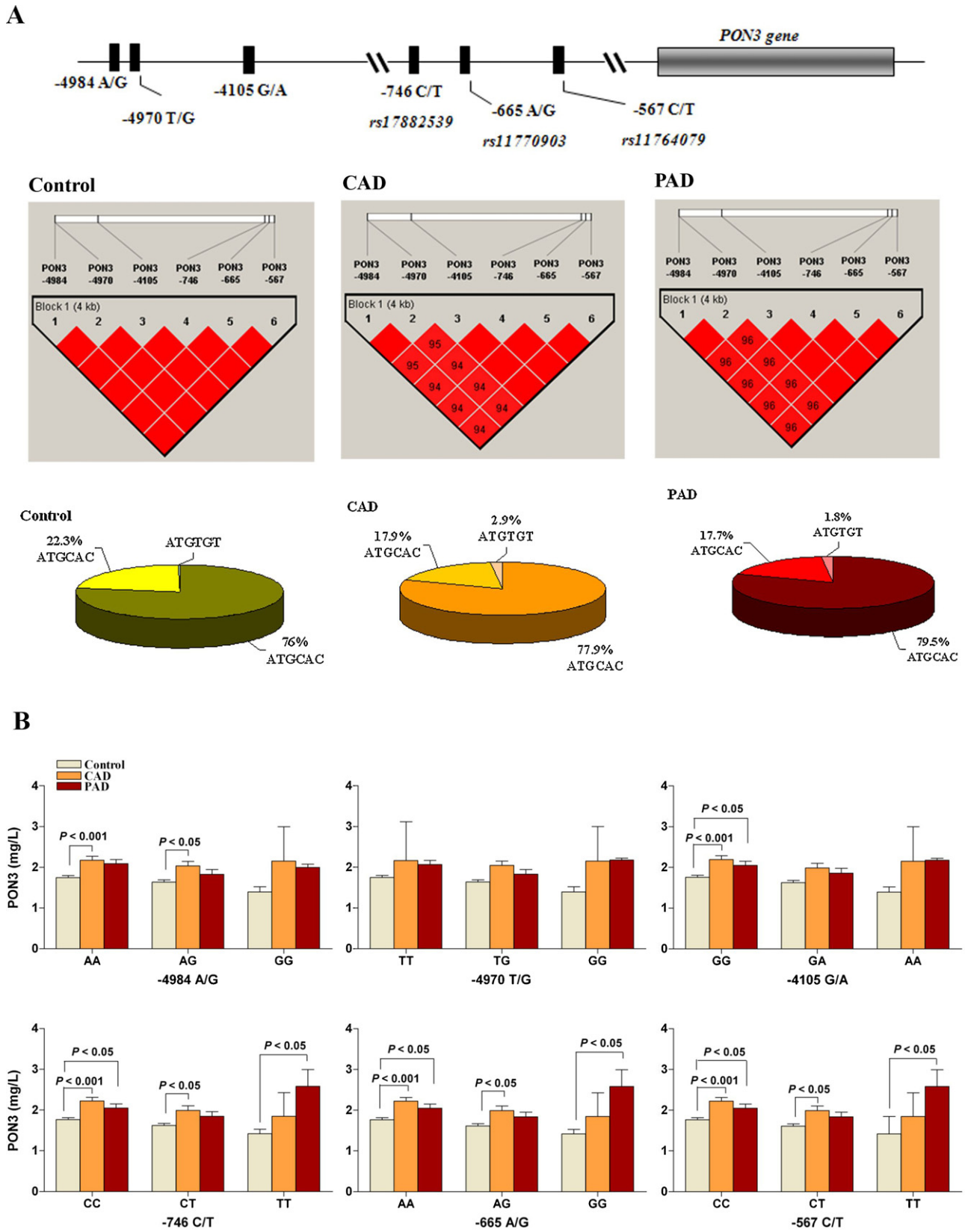


Fig. 4. (A) Linkage disequilibrium structures across the *PON3* promoter gene, in control subjects, patients with coronary artery disease (CAD) and patients with peripheral artery disease (PAD), calculated by the Haploview software and labelled by their gene position. Pairwise linkage disequilibrium values are given in each square intersecting for each pair of SNPs and haplotype blocks outlined. There were not any significant differences in the haplotype frequencies between patients and controls. (B) Influence of the *PON3* gene promoter polymorphisms on serum PON3 concentrations in the three groups of subjects studied.

Table 1
Selected biochemical variables in patients with coronary artery disease (CAD) and peripheral artery disease (PAD) compared with the control group.

Variable	Controls ($n=175$)	CAD ($n=72$)	PAD ($n=118$)	*P*-value CAD vs control	*P*-value PAD vs control	*P*-value PAD vs CAD
Total cholesterol, mmol/L	5.45 (0.07)	4.40 (0.15)	3.98 (0.09)	<0.001	<0.001	0.015
HDL-cholesterol, mmol/L	1.53 (0.03)	1.18 (0.09)	1.08 (0.03)	<0.001	<0.001	1.000
LDL-cholesterol, mmol/L	3.33 (0.07)	2.68 (0.12)	2.30 (0.09)	<0.001	<0.001	0.006
Triglycerides, mmol/L	1.32 (0.06)	1.45 (0.13)	2.98 (0.10)	0.762	<0.001	<0.001
Glucose, mmol/L	5.20 (0.11)	6.44 (0.29)	6.55 (0.25)	<0.001	<0.001	0.831
Insulin, pmol/L	53.22 (2.47)	78.04 (6.59)	114.71 (11.17)	<0.001	<0.001	0.283
HOMA-IR	0.99 (0.04)	1.54 (0.13)	2.19 (0.20)	<0.001	<0.001	0.337
PON1 concentration, mg/L	117.95 (6.84)	57.16 (2.09)	32.02 (1.43)	<0.001	<0.001	<0.001
PON3 concentration, mg/L	1.69 (0.04)	2.12 (0.07)	1.98 (0.08)	<0.001	0.05	0.011
Lactonase activity, U/L	5.85 (0.13)	5.91 (0.21)	3.31 (0.14)	0.420	<0.001	<0.001
Paraoxonase activity, U/L	322.78 (9.53)	331.23 (14.50)	197.94 (9.66)	0.528	<0.001	<0.001
β2M, mg/L	0.9 (0.4)	1.88 (0.08)	4.15 (0.28)	<0.001	<0.001	<0.001
CCL2, ng/L	458.84 (29.73)	430.66 (12.38)	656.33 (320.33)	0.555	<0.001	<0.001
High-sensitivity CRP, mg/L	2.42 (0.36)	4.95 (1.13)	58.16 (7.33)	<0.001	<0.001	<0.001

Values are given as mean ± SEM or percentages.

of lipoprotein aggregates in the intima, and monocyte migration through the endothelium and their differentiation into macrophages [23]. Lipid peroxidation is linked to the progression of the disease from fatty streak to advanced atherosclerotic plaque [24]. Both PAD and CAD present with common biochemical characteristics linked to the onset and progression of atherosclerosis, but the extent of the diseased area in PAD is considerably higher to that in CAD. For this reason, it would be logical to expect qualitative and quantitative differences in the molecular factors involved in the progression of these diseases [25]. Manifestation of arteriosclerosis in lower extremity arteries may show characteristics which are not seen in other clinical events and to explore the similarities and differences between CAD and PAD may be clinically relevant. For instance, increase in hs-CRP and β2M concentrations is quantitatively much more important in patients with PAD than in patients with CAD, showing that the inflammatory response is extreme in these patients, which is probably related to the physical extension of this disease [26]. We recently reported that serum PON1 lactonase and paraoxonase activities, as well as concentration, are dramatically decreased in patients with PAD compared to controls, suggesting that this enzyme may be involved in the biochemical derangements leading to this disease [18].

In the present study we describe, for the first time, alterations in serum PON3 concentrations in patients with coronary and with peripheral atherosclerosis. Our patients with CAD or PAD had a significantly increased serum PON3 concentrations with respect to the control group which was accompanied by decreased PON1 concentrations (and activities in PAD) and decreased HDL-cholesterol levels. Although the increase in PON3 is quantitatively small, about 1 mg/L on average, compared to the 50–70 mg/L decrease in PON1, it has to be taken into account that PON3 is about 100 times more potent per mg of protein than PON1 in protecting LDL against lipid peroxidation [27] and, thus, the increase in the enzyme's expression in these patients would be sufficient to balance the alterations in PON1 levels. This conclusion is in agreement with experimental studies showing that the overexpression of human PON3 in mice protected against the progression of atherosclerosis in mice [28]. It is to note, however, that PON1 activity is essentially normal in CAD patients and extremely low in PAD. This is important, due to the wide prescription of clopidogrel in these patients and, as recently suggested, the need for PON1 for its activation [29]. Whether this fact is relevant for clinical management of patients with PAD or CAD remains to be ascertained [30].

An interesting finding of the present article is the observed relationship between serum PON3 concentrations, hypertension and metabolic alterations in PAD, and particularly with insulin and the HOMA index, that was not found in subjects with CAD. Diabetes is the most common underlying disease causing PAD, and these results suggest a role for PON3 in the regulation of glucose metabolism, which is one of the most important derangements related to metabolic syndrome. Indeed, human PON3 transgenic mice have been reported to have lower insulin concentrations than their corresponding littermates and are protected against obesity development [31]. In PAD, however, serum PON3 concentrations were not associated to circulating inflammatory markers as β2M, CCL2 or hs-CRP, an association that was positively observed in CAD. We do not know the reason for these differences, but serum PON3 concentrations were significantly lower in PAD, while the levels of inflammatory markers were much higher. The possibility exists that the magnitude of the physical extent of the diseased areas in PAD makes for PON3 impossible to thwart the massive arterial inflammation observed in these patients. We also analysed *PON3* gene promoter polymorphisms, to ascertain whether the observed differences in serum PON3 concentrations could be explained by differences in the gene frequency distribution between patients and controls. We did not find any significant differences in these polymorphisms and their haplotypes, and their influence on serum PON3 concentrations was similar in the three groups of subjects.

5. Conclusion

PON3 is still a relatively newly identified antioxidant enzyme and much work has yet to be done in order to elucidate its physiological functions and its implications in atherosclerosis. The present cross-sectional, pilot study found that serum PON3 concentration is increased in patients with CAD or PAD, two different manifestations of atherosclerosis with clearly differentiated phenotypes. We observed that PON3 seems to be more narrowly related to insulin sensitivity in PAD and to inflammation in CAD. However, further detailed studies would be necessary to fully understand the role of PON3 in human atherosclerosis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

Acknowledgments

We thank Dr. John Teiber (University of Texas, Dallas, TX) and Dr. Dan Tawfik (Weizmann Institute of Science, Rehovot, Israel) for the generous gifts of purified PON3 and the TBBL reagent, respectively. This study was supported by grants from the *Instituto de Salud Carlos III* (PI 08/1175, and PI08/1381), Madrid, Spain, and the *Fondo*

Europeo de Desarrollo Regional (FEDER). Anna Rull is the recipient of a fellowship from the Generalitat de Catalunya (FI-G0503).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.atherosclerosis.2011.11.021.

References

[1] Seo D, Goldschmidt-Clermont P. The paraoxonase gene family and atherosclerosis. Curr Atheroscler Rep 2009;11:182–7.
[2] Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. Genomics 1996;33:498–507.
[3] Rodríguez-Sanabria F, Rull A, Beltrán-Debón R, et al. Tissue distribution and expression of paraoxonases and chemokines in mouse: the ubiquitous and joint localisation suggest a systemic and coordinated role. J Mol Histol 2010;41:379–86.
[4] Deakin SP, Bioletto S, Bochaton-Piallat ML, James RW. HDL-associated paraoxonase-1 can redistribute to cell membranes and influence sensitivity to oxidative stress. Free Rad Biol Med 2011;50:102–9.
[5] Ng CJ, Wadleigh DJ, Gangopadhyay A, et al. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. J Biol Chem 2001;76:44444–9.
[6] Camps J, Marsillach J, Joven J. The paraoxonases: role in human diseases and methodological difficulties in measurement. Crit Rev Clin Lab Sci 2009;46:83–106.
[7] Bourquard N, Shih DM, Ng CJ, et al. The role of PON2 and PON3 in atherosclerosis and related traits. In: Mackness B, Mackness M, Aviram M, Paragh G, editors. The paraoxonases: their role in disease development and xenobiotic metabolism. Dordrecht: Springer; 2008. p. 103–28.
[8] Aragonès G, Guardiola M, Barreda M, et al. Measurement of serum paraoxonase-3 concentration: method evaluation, reference values and influence of genotypes in a population-based study. J Lipid Res 2011;52:1055–61.
[9] Yun WS, Rho YN, Park UJ, Lee KB, Kim DI, Kim YW. Prevalence of asymptomatic critical carotid artery stenosis in Korean patients with chronic atherosclerotic lower extremity ischemia: is a screening carotid duplex ultrasonography worthwhile? J Korean Med Sci 2010;25:1167–70.
[10] Fowkes FG, Murray GD, Butcher I, et al. Ankle brachial index combined with Framingham risk score to predict cardiovascular events and mortality: a meta-analysis. JAMA 2008;300:197–208.
[11] Bertrán N, Camps J, Fernández-Ballart J, et al. Diet and lifestyle are associated with serum C-reactive protein concentrations in a population-based study. J Lab Clin Med 2005;145:41–6.
[12] Marsillach J, Mackness B, Mackness M, et al. Immunochemical analysis of paraoxonases-1, 2, and 3 expression in normal mouse tissues. Free Radic Biol Med 2008;45:146–57.
[13] Gaidukov L, Tawfik D. The development of human sera tests for HDL-bound serum PON1 and its lipolactonase activity. J Lipid Res 2007;48:1637–46.
[14] Marsillach J, Aragonès G, Beltrán R, et al. The measurement of the lactonase activity of paraoxonase-1 in the clinical evaluation of patients with chronic liver impairment. Clin Biochem 2009;42:91–8.
[15] Ferré N, Camps J, Prats E, et al. Serum paraoxonase activity: a new additional test for the improved evaluation of chronic liver damage. Clin Chem 2002;48:261–8.
[16] Tanno K, Okamura T, Ohsawa M, et al. Comparison of low-density lipoprotein cholesterol concentrations measured by a direct homogeneous assay and by the Friedewald formula in a large community population. Clin Chim Acta 2010;411:1774–80.
[17] Matthews DR, Hosker J, Rudenski A, Naylor B, Treacher D, Turner R. Homeostasis model assessment: insulin resistance and B-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.
[18] Rull A, García R, Fernández-Sender L, et al. The role of combined assessment of defense against oxidative stress and inflammation in the evaluation of peripheral arterial disease. Curr Mol Med 2011;11:453–64.
[19] Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263–5.
[20] Ross R. Cell biology of atherosclerosis. Annu Rev Physiol 1995;57:791–804.
[21] Aviram M. Atherosclerosis: cell biology and lipoproteins – inflammation and oxidative stress in atherogenesis: protective role for paraoxonases. Curr Opin Lipidol 2011;22:243–4.
[22] Rosenblat M, Aviram M. Paraoxonases role in the prevention of cardiovascular diseases. Biofactors 2009;35:98–104.
[23] Tavori H, Vaya J, Aviram M. Paraoxonase 1 attenuates human plaque atherogenicity: relevance to the enzyme lactonase activity. Adv Exp Med Biol 2010;660:99–111.
[24] Aviram M, Rosenblat M. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. Free Radic Biol Med 2004;37:1304–16.
[25] Brevetti M, Piscione F, Cirillo P, et al. In concomitant coronary and peripheral arterial disease, inflammation of the affected limbs predicts coronary artery endothelial dysfunction. Atherosclerosis 2008;201:440–6.
[26] Wilson AM, Kimura E, Harada RK, et al. Beta2-microglobulin as a biomarker in peripheral arterial disease: proteomic profiling and clinical studies. Circulation 2007;116:1396–403.
[27] Draganov DI, Stetson PI, Watson CE, Billecke SS, La Du BN. Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. J Biol Chem 2000;275:33435–42.
[28] Ng CJ, Bourquard N, Hama SY, et al. Adenovirus-mediated expression of human paraoxonase 3 protects against the progression of atherosclerosis in apolipoprotein E-deficient mice. Atheroscler Thromb Vasc Biol 2007;27:1–7.
[29] Bouman HJ, Schömig E, van Werkum JW, et al. Paraoxonase-1 is a major determinant of clopidogrel efficacy. Nat Med 2011;17:110–6.
[30] Camps J, Joven J, Mackness B, et al. Paraoxonase-1 and clopidogrel efficacy. Nat Med 2011;17:1041–2.
[31] Shih DM, Xia YR, Wang XP, et al. Decreased obesity and atherosclerosis in human paraoxonase 3 transgenic mice. Circ Res 2007;100:1200–7.

IV *DISCUSIÓN Y CONCLUSIONES*

Dipòsit Legal: T. 191-2013

El estrés oxidativo y la inflamación juega un papel clave en la arterosclerosis. La formación de placa aterosclerótica y su progresión se caracteriza por una disfunción de las células endoteliales, la acumulación de agregados de las lipoproteínas en la íntima, y a la migración de monocitos a través del endotelio y su diferenciación en macrófagos. La peroxidación de los lípidos está relacionada con la progresión de la enfermedad de la estría grasa a la placa aterosclerótica avanzada. Sin embargo, los pacientes con EAP sintomática representan una condición en la cual la extensión en múltiples vasos puede garantizar una alta carga de aterosclerosis, mayor que en los pacientes con CAD, y es probable que se presenten diferencias cualitativas y cuantitativas de los factores moleculares implicados en la progresión de la enfermedad. Por ejemplo, el aumento de PCR ultrasensible y las concentraciones de β2M son cuantitativamente mucho más importantes en los pacientes con EAP que en los pacientes con CAD, que muestran que la respuesta inflamatoria es extrema en estos pacientes, y que probablemente está relacionada con la extensión física de esta enfermedad.

Exploramos por primera vez, si la relación inversa entre los mecanismos de aceptación general de defensa contra el estrés oxidativo del endotelio y la consiguiente respuesta inflamatoria se puede detectar en la sangre. Hemos encontrado que los resultados obtenidos de MCP-1 plasmático y las concentraciones séricas de PON1, así como PON1 paraoxonasa y lactonasa prueba nuestra hipótesis y pueden constituir un nuevo indicador del estado de la enfermedad que pueden actuar como biomarcadores.

La disminución de las actividades paraoxonasa y lactonasa se asociaron significativamente con un aumento de las concentraciones circulantes de MCP-1. Sin embargo, la interpretación de MCP-1 plasmático es difícil. Aunque en otras condiciones la concentración plasmática de MCP-1 fue independiente de otras variables inflamatorias, se ha mostrado una buena correlación con los niveles plasmáticos de PCR y β2M, confirmando parcialmente los efectos in vitro de ciertas moléculas que circulan por la expresión de quimioquinas en las células endoteliales humanas.

Por tanto, proponemos el uso en combinación de ambos, el mayor MCP-1 asociado con un menor PON1, especialmente el ratio entre MCP-1 y PON1, que distingue claramente a los pacientes de los controles y muestra un fuerte, casi completo valor predictivo.

A nivel celular PON1 parece modular la producción de MCP-1, pero ambos son distribuidos por doquier en la mayoría de los tejidos. Por otra parte, en los vasos enfermos, la expresión de ambas proteínas es mayor.

Nuestros resultados en los pacientes con CAD, con posiblemente una menor carga de aterosclerosis, también se sugiere un papel de coordinación de PON1 y MCP-1 y, posiblemente, de una relación cuantitativa. Contrariamente a los hallazgos en pacientes con enfermedad arterial periférica, no se encontraron cambios significativos en el MCP-1 plasmático o PON1 lactonasa con respecto a los controles y valores sólo ligeramente inferiores de la actividad de la paraoxonasa PON1, pero la concentración circulante de PON1 fue menor, mostrando un posible valor predictivo del ratio de MCP1 con PON1 en la extensión de la arteriosclerosis.

En conclusión, nuestros resultados clínicamente identificar una relación inversa entre MCP-1 y PON1, que es independiente de las variaciones genéticas y que la relación entre la defensa contra el estrés oxidativo y la inflamación puede ser detectado mediante simples mediciones de laboratorio en pacientes con aterosclerosis.

En el segundo estudio se describe, por primera vez, las alteraciones en las concentraciones séricas de PON3 en pacientes con CAD y con aterosclerosis periférica. Nuestros pacientes con enfermedad coronaria o arterial periférica tenían un aumento significativo de las concentraciones en el suero PON3 que fue acompañado por una disminución de las concentraciones de PON1 (y actividades en PAD) y la disminución de niveles de colesterol HDL. Esto implica un enriquecimiento relativo de las partículas de HDL en PON3 en estos temas, que es, posiblemente, un mecanismo compensatorio para contrarrestar la disminución de PON1. Esta conclusión está de acuerdo con los estudios experimentales que muestran que la sobreexpresión de PON3 humana en ratones protege contra la progresión de la aterosclerosis en ratones.

Un hallazgo interesante del presente artículo es la relación observada entre las concentraciones séricas PON3, hipertensión arterial y alteraciones metabólicas en la EAP, y en particular con la insulina y el índice HOMA, que no se ha encontrado en pacientes con CAD. En la EAP, las concentraciones séricas de PON3 no fueron asociados a marcadores inflamatorios circulantes como β2M, CCL2 o hs-CRP, una asociación que se observó de manera positiva en el CAD. No sabemos la razón de estas diferencias, pero las concentraciones séricas PON3 fueron significativamente menores en la EAP, mientras que los niveles de marcadores inflamatorios fueron mucho más altos. También se investigó la posibilidad de una asociación entre la aterosclerosis y PON3 polimorfismos de promotor del gen. No se encontró ninguna diferencia significativa en estos polimorfismos y sus haplotipos entre pacientes y

controles, y su influencia en las concentraciones séricas de PON3 fue similar en los tres grupos de sujetos. PON3 sigue siendo una enzima antioxidante relativamente recientemente identificados y mucho trabajo aún no se ha hecho con el fin de aclarar sus funciones y sus implicaciones en la aterosclerosis. El estudio piloto encontró que la concentración sérica de PON3 es mayor en pacientes con enfermedad coronaria o arterial periférica, dos enfermedades diferentes asociados con la aterosclerosis, pero con algunas diferencias en las alteraciones bioquímicas subyacentes. Hemos observado que PON3 parece estar más estrechamente relacionados con la resistencia a la insulina en la EAP y la inflamación en el CAD.

Dipòsit Legal: T. 191-2013

-La relación entre defensa, estrés oxidativo e inflamación se puede detectar mediante pruebas analíticas de laboratorio

-Existe una relación inversa entre MCP-1 y PON1 que es independiente de variaciones genéticas.

-La concentración sérica de PON3 está aumentada en pacientes con coronariopatías (asociado a incremento de parámetros inflamatorios) y en pacientes con enfermedad arterial periférica (asociado a sensibilidad a la insulina).

Dipòsit Legal: T. 191-2013

V BIBLIOGRAFÍA

1. TASC II. Management of peripheral arterial disease (PAD). TransAtlantic Inter-Society Consensus (TASC II). Eur J Vasc Endovasc Surg 2007;33,S1-S75.

2. Clement DL, Boccalon H, Dormandy J, Durand-Zaleski I, Fowkes G, Brown T. A clinical approach to the management of the patient with coronary (Co) and/or carotid (Ca) artery disease who presents with leg ischaemia (Lis). Int Angiol 2000;19(2):97-125.

3. AHCPR. United States Department of Health and Human Services. Agency for Health Care Policy and Research. Acute pain management: operative on medical procedures and trauma. [107]. Rockville MD: AHCPR; 1993.

4. Criqui MH, Fronek A, Barrett-Connor E, Klauber MR, Gabriel S, Goodman D. The prevalence of peripheral arterial disease in a defined population. Circulation 1985;71(3):510-551.

5. Hiatt WR, Hoag S, Hamman RF. Effect of diagnostic criteria on the prevalence of peripheral arterial disease. The San Luis Valley Diabetes Study. Circulation 1995;91(5):1472-1479.

6. Selvin E, Erlinger TP. Prevalence of and risk factors for peripheral arterial disease in the United States: results from the National Health and Nutrition Examination Survey, 1999-2000. Circulation 2004;110(6):738-743.

7. Fowkes FG, Housley E, Cawood EH, Macintyre CC, Ruckley CV, Prescott RJ. Edinburgh Artery Study: prevalence of asymptomatic and symptomatic peripheral arterial disease in the general population. Int J Epidemiol 1991;20(2):384-392.

8. Hirsch A, Criqui M, Treat-Jacobson D, Regensteiner J, Creager M, Olin J, et al. Peripheral arterial disease detection, awareness, and treatment in primary care. JAMA 2001;286(11):1317-1324.

9. McDermott MM, Criqui MH, Greenland P, Guralnik JM, Liu K, Pearce WH, et al. Leg strength in peripheral arterial disease: associations with disease severity and lower-extremity performance. J Vasc Surg 2004;39(3):523-530.

10. Widmer L, Biland L. Risk profile and occlusive peripheral arterial disease. Proceedings of 13th International Congress of Angiology 1985:28.

11. Kannel WB, Skinner JJ, Jr., Schwartz MJ, Shurtleff D. Intermittent claudication. Incidence in the Framingham Study. Circulation 1970;41(5):875-883.

12. Rothwell PM, Eliasziw M, Gutnikov SA, Warlow CP, Barnett HJ. Endarterectomy for symptomatic carotid stenosis in relation to clinical subgroups and timing of surgery. Lancet 2004;363(9413):915-924.

13. Tunis SR, Bass EB, Steinberg EP. The use of angioplasty, bypass surgery, and amputation in the management of peripheral vascular disease. N Engl J Med 1991;325(8):556-562.

14. Dormandy J, Belcher G, Broos P, Eikelboom B, Laszlo G, Konrad P, et al. Prospective study of 713 below-knee amputations for ischaemia and the effect of a prostacyclin analogue on healing. Hawaii Study Group. Br J Surg 1994;81(1):33-37.

15. Bhatt D, Steg P, Ohman E, Hirsch A, Ikeda Y, Mas J, et al. International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. JAMA 2006;295:180-189.

16. Aronow WS, Ahn C. Prevalence of coexistence of coronary artery disease, peripheral arterial disease, and atherothrombotic brain infarction in men and women > or = 62 years of age. Am J Cardiol 1994;74(1):64-65.

17. Dormandy JA, Murray GD. The fate of the claudicant--a prospective study of 1969 claudicants. Eur J Vasc Surg 1991;5(2):131-133.

18. Mehler PS, Coll JR, Estacio R, Esler A, Schrier RW, Hiatt WR. Intensive blood pressure control reduces the risk of cardiovascular events in patients with peripheral arterial disease and type 2 diabetes. Circulation 2003;107(5):753-756.

19. Criqui M, Langer R, Fronek A, Feigelson H, Klauber M, McCann T, et al. Mortality over a period of 10 years in patients with peripheral arterial disease. N Engl J Med 1992;326:381-386.

20. Fowkes F, Lee A, Murray G. on behalf of the ABI collaboration. Anklebrachial index as an independent indicator of mortality in fifteen international population cohort studies. Circulation 2005;112:3704.

21. CAPRIE. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet 1996;348(9038):1329-1339.

22. Resnick HE, Lindsay RS, McDermott MM, Devereux RB, Jones KL, Fabsitz RR, et al. Relationship of high and low ankle brachial index to all-cause and cardiovascular disease mortality: the Strong Heart Study. Circulation 2004;109(6):733-739.

23. Gerard C, Rollins BJ. Chemokines and disease. *Nat Immunol.* 2001; 2: 108–115.

24. Luster A D. Chemokines-Chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998 ; 338 (7): 436-45.

25.Charo IF, Taubman MB. Chemokines in the pathogenesis of vascular disease. *Circ Res* 2004; 95(9):858-66.

26. Huo Y, Weber C, Forlow SB, Sperandio M, Thatte J, Mack M, Jung S, Littman DR, Ley K. The chemokine KC, but not monocyte chemoattractant protein-1, triggers monocyte arrest on early atherosclerotic endothelium.*J Clin Invest.*2001;108:1307–1314.

27. Gerszten RE, Garcia-Zepeda EA, Lim Y-C, Yoshida M, Ding HA, Gimbrone MA, Jr., Luster AD, Luscinskas FW, Rosenzweig A. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions.*Nature.*1999;398:718–723.

28. Gerszten RE, Lim Y-C, Ding HT, Snapp K, Kansas G, Dichek DA, Cabañas C, Sánchez-Madrid F, Gimbrone MA, Jr, Rosenzweig A, Luscinskas FW. Adhesion of monocytes to vascular cell adhesion molecule-1-transduced human endothelial cells. Implications for atherogenesis.*Circ Res.*1998;82:871–878.

29. Bazan JF, Bacon KB, Hardiman G, Wang W, Soo K, Rossi D, Greaves DR, Zlotnik A, Schall TJ. A new class of membrane-bound chemokine with a CX_3C motif.*Nature.*1997;385:640–644.

30. Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo J-A, Vath J, Gosselin M, Ma J, Dussault B, Woolf E, Alperin G, Culpepper J, Gutierrez-Ramos JC, Gearing D. Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation.*Nature.*1997;387:611–617.

31. Haskell CA, Cleary MD, Charo IF. Molecular uncoupling of fractalkine-mediated cell adhesion and signal transduction. Rapid flow arrest of CX_3CR1-expressing cells is independent of G-protein activation.*J Biol Chem.*1999;274:10053–10058.

32. Fong AM, Robinson LA, Steeber DA, Tedder TF, Yoshie O, Imai T, Patel DD. Fractalkine and CX_3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow*J Exp Med.*1998;188:1413–1419.

33. Tsou C-L, Haskell CA, Charo IF. Tumor necrosis factor-α-converting enzyme mediates the inducible cleavage of fractalkine.*J Biol Chem.*2001;276:44622–44626.

34. Matloubian M, David A, Engel S, Ryan JE, Cyster JG. A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo.*Nat Immunol.*2000;1:298–304.

35. Shimaoka T, Kume N, Minami M, Hayashida K, Kataoka H, Kita T, Yonehara S. Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages.*J Biol Chem.*2000;275:40663–40666.

36. Xu J, Wang F, Van Keymeulen A, Herzmark P, Straight A, Kelly K, Takuwa Y, Sugimoto N, Mitchison T, Bourne HR. Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils.*Cell.*2003;114:201–214.

37. Wiener OD, Neilsen PO, Prestwich GD, Kirschner MW, Cantley LC, Bourne HR. A $PtdInsP_3$- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity.*Nat Cell Biol.*2002;4:509–512.

38. Gerrity RG, Naito HK. Ultrastructural identification of monocyte-derived foam cells in fatty streak lesions.*Artery.*1980;8:208–214.

39. Faggiotto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation.*Arteriosclerosis.*1984;4:323–340.

40. Nelken NA, Coughlin SR, Gordon D, Wilcox JN. Monocyte chemoattractant protein-1 in human atheromatous plaques.*J Clin Invest.*1991;88:1121–1127.

41. Yu X, Dluz S, Graves DT, Zhang L, Antoniades HN, Hollander W, Prusty S, Valente AJ, Schwartz CJ, Sonenshein GE. Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates.*Proc Natl Acad Sci U S A.*1992;89:6953–6957.

42. Steinberg D. Lewis A. Conner Memorial Lecture. Oxidative modification of LDL and atherogenesis.*Circulation.*1997;95:1062–1071.

43. Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells.*Proc Natl Acad Sci U S A.*1990;87:5134–5138.

44. Grewal IS, Rutledge BJ, Fiorillo JA, Gu L, Gladue RP, Flavell RA, Rollins BJ. Transgenic monocyte chemoattractant protein-1 (MCP-1) in pancreatic islets produces monocyte-rich insulitis without diabetes. Abrogation by a second transgene expressing systemic MCP-1 *J Immunol.*1997;159:401–408.

45. Aiello RJ, Bourassa P-AK, Lindsey S, Weng W, Natoli E, Rollins BJ, Milos PM. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice.*Arterioscler Thromb Vasc Biol.*1999;19:1518–1525.

46. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ. Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice *Mol Cell.*1998;2:275–281.

47. Gosling J, Slaymaker S, Gu L, Tseng S, Zlot CH, Young SG, Rollins BJ, Charo IF. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B.*J Clin Invest.*1999;103:773–778.

48. Boring L, Gosling J, Cleary M, Charo IF. Decreased lesion formation in $CCR2^{-/-}$ mice reveals a role for chemokines in the initiation of atherosclerosis.*Nature.*1998;394:894–897.

49. Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. The human serum paraoxonase/arylesterase gene *(PON1)* is one member of a multigene family. *Genomics* 1996; 33: 498–507.

50. Sorenson RC, Primo-Parmo SL, Camper SA, La Du BN. The genetic mapping and gene structure of mouse paraoxonase/ arylesterase. *Genomics* 1995; 30: 431–438.

51. La Du BN. Human serum paraoxonase/arylesterase. In Kalow W, Ed. *Pharmacogenetics of Drug Metabolism.* Pp 51– 91. New York: Pergamon Press, 1992.

52. Leviev I, Negro F, James RW. Two alleles of the human paraoxonase gene produce different amounts of mRNA. *Arterioscler Thromb Vasc Biol* 1997; 17: 2935–2939.

53. Sierksma A, van der Gaag MS, van Tol A, James RW, Hendriks FJ. Kinetics of HDL cholesterol and paraoxonase activity in moderate alcohol consumers. *Alcohol Clin Exp Res* 2002; 26: 1430–1435.

54. Jaouad L, de Guise C, Berrougui H, Cloutier M, Isabelle M, Fulop T, Pavette H, Khalil A. Age-related decreased in highdensity lipoproteins antioxidant activity is due to an alteration in the PON1s free sulfhydryl groups. *Atherosclerosis* 2006; 185: 191–200.

55. Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM, Reddy ST. Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *J Biol Chem* 2001; 276: 44444–44449.

56. Billecke S, Draganov D, Counsell R, Stetson P, Watson C, Hsu C, La Du BN. Human serum paraoxonase (PON1) isozymes Q and R hydrolyze lactones and cyclic carbonate esters. *Drug Metab Dispos* 2000; 28: 1335–1342.

57. Costa LG, Cole TB, Vitalone A, Furlong CE. Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin Chim Acta* 2005; 352: 37–47.

58. Draganov DI, Teiber JF, Speelman A, Osawa Y, Sunahara R, La Du BN. Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J Lipid Res* 2005; 46: 1239–1247.

59. Aviram M, Rosenblat M. Paraoxonases 1, 2, and 3, oxidative stress, and macrophage cell formation during atherosclerosis development. *Free Rad Biol Med* 2004; 37: 1304–1316.

60. Horke S, Witte I, Wilgenbus P, Krüger M, Strand D, Förstermann U. Paraoxonase-2 reduces oxidative stress in vascular cells and decreases endoplasmic reticulum stress-induced caspase activation. *Circulation* 2007; 115:2055–2064.

61. Mackness B, Quarck R, Verreth W, Mackness M, Holvoet P. Human paraoxonase-1 overexpression inhibits atherosclerosis in a mouse model of metabolic syndrome. *Arterioscler Thromb Vasc Biol* 2006; 26: 1545–1550.

62. Ng CJ, Hama SY, Bourquard N, Navab M, Reddy ST. Adenovirus mediated expression of human paraoxonase 2 protects against the development of atherosclerosis in apolipoprotein E-deficient mice. *Mol Genet Metab* 2006; 89: 368–373.

63. Shih DM, Xia YR, Wang XP, Wang SS, Bourquard N, Fogelman AM, Lusis AJ, Reddy ST. Decreased obesity and atherosclerosis in human paraoxonase 3 transgenic mice. *Circ Res* 2007; 100: 1200–1207.

64. Ng CJ, Bourquard N, Hama SY, Shih D, Grijalva VR, Navab M, Fogelman AM, Reddy ST. Adenovirus-mediated expression of human paraoxonase 3 protects against the progression of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2007; 27: 1368–1374.

65. Aldridge WN. Serum esterases I. Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate and a method for their determination. *Biochem J* 1953; 53: 110–117.

66. Navab M, Ananthramaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fonarow GC, Vahabzadeh K, Hama S, Hough G, Kamranpour N, Berliner JA, Lusis AJ, Fogelman AM. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. *J Lipid Res* 2004; 45: 993–1007.

67. Navab M, Hama SY, Cooke CJ, Anantharamaiah GM, Chaddha M, Jin L, Subbanagounder G, Faull KF, Reddy ST, Miller NE, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J Lipid Res* 2000; 41: 1481–1484.

68. Navab M, Hama SY, Anantharamaiah GM, Hassan K, Hough GP, Watson AD, Reddy ST, Sevanian A, Fonarow GC, Fogelman AM. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: steps 2 and 3. *J Lipid Res* 2000; 41: 1485–1508.

69. Negre-Salvayre A, Dousset N, Ferretti G, Bacchetti T, Curatola G, Salvayre R. Antioxidant and cytoprotective properties of high-density lipoproteins in vascular cells. *Free Rad Biol Med* 2006; 41: 1031–1040.

70. Clay MA, Pyle DH, Rye KA, Vadas MA, Barter PJ. Time sequence of inhibition of endothelial adhesion molecule expression by reconstituted high density lipoproteins. *Atherosclerosis* 2001; 157: 23–29.

71. Cockerill GW, Rye KA, Gamble JR, Vadas MA, Barter PJ. High density lipoproteins inhibit cytokine induced expression of endothelial cell adhesion molecules. *Arterioscler Thromb Vasc Biol* 1995; 15: 1987–1994.

72. Calabresi L, Gomaraschi M, Vilia B, Omobani L, Dmitrieff C, Franceschini GE. Elevated soluble adhesion molecules in subjects with low HDL-cholesterol. *Arterioscler Thromb Vasc Biol* 2002; 22: 656–661.

73. Birjmohun RS, Van Leuven SI, Levels JHM, Van't Veer C, Kuivenhoven JA, meijers JCM, Levi M, Kastelein JJP, Van der Poll T, Stroes ESG. High-density lipoprotein attenuates inflammation and coagulation on endotoxin challenge in humans. *Arterioscler Thromb Vasc Biol* 2007; 27: 1153–1158.

74. Simpson KJ, Henderson NC, Bone-Larson CL, Lukacs NW, Hogaboam CM, Kunkel SL. Chemokines in the pathogenesis of liver disease: so many players with poorly defined roles. *Clin Sci (Lond)* 2003; 104: 47–63.

75. Navab M, Imes SS, Hama SY, Hough GP, Ross LA, Bork RW, Valente AJ, Berliner JA, Drinkwater DC, Laks H, Fogelman AM. Monocyte transmigration induced by modification of low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein 1 synthesis and is abolished by high density lipoprotein. *J Clin Invest* 1991; 88: 2039–2046.

76. Watson AD, Berliner JA, Hama SY, La Du BN, Fault KF, Fogelman AM, Navab M. Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidised low-density lipoprotein. *J Clin Invest* 1995; 96: 2882–2891.

77. Mackness B, Hine D, Liu Y, Mastorikou M, Mackness M. Paraoxonase-1 inhibits oxidised LDL-induced MCP-1 production by endothelial cells. *Biochem Biophys Res Commun* 2004; 318: 680–683.

78. Tward A, Xia YR, Wang XP, Shi YS, Park C, Castellani LW, Lusis AJ, Shih DM. Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation* 2002; 106: 484–490.

79. Rozenberg O, Rosenblat M, Coleman R, Shih DM, Aviram M. Paraoxonase (PON1) deficiency is associated with increased macrophage oxidative stress: studies in PON1-knockout mice. *Free Rad Biol Med* 2003; 34: 774–784.

80. Cabana VG, Reardon CA, Feng N, Neath S, Lukens J, Getz GS. Serum paraoxonase: effect of the apolipoprotein composition of HDL and the acute phase response. *J Lipid Res* 2003; 44: 780–792.

81. Bergmeier C, Siekmeier R, Gross W. Distribution spectrum of paraoxonase activity in HDL fractions. *Clin Chem* 2004; 50: 2309–2315.

82. Ribas V, Sánchez-Quesada J, Antón R, Camacho M, Julve J, Escolà-Gil JC, Vila L, Ordóñez-Llanos J, Blanco-Vaca F. Human apolipoprotein A-II enrichment displaces paraoxonase from HDL and impairs its antioxidant properties. A new mechanism linking HDL protein composition and antiatherogenic potential. *Circ Res* 2004; 95: 789–797.

83. Feingold KR, Memon RA, Mosser AH, Grunfeld C. Paraoxonase activity in the serum and hepatic mRNA levels decrease during the acute phase response. *Atherosclerosis* 1998; 139: 307–315.

84. Bin Ali A, Zhang Q, Lim YK, Fang D, Retnam L, Lim SK. Expression of major HDL-associated antioxidant PON-1 is gender dependent and regulated during inflammation. *Free Rad Biol Med* 2003; 34: 824–829.

85. Han CY, Chiba T, Campbell JS, Fausto N, Chaisson M, Orasanu G, Plutzky J, Chait A. Reciprocal and coordinate regulation of serum amyloid A versus apolipoprotein A-I and paraoxonase-1 by inflammation in murine hepatocytes. *Arterioscler Thromb Vasc Biol* 2006; 26: 1806–1813.

86. Bourquard N, Shih DM, Ng CJ, Villa-García N, Nakamura K, Stoltz DA, Ozer E, Grijalva V, Rozengurt N, Hama SY, Zabner J, Navab M, Fogelman AM, Reddy ST. The role of PON2 and PON3 in atherosclerosis and related traits. In Mackness B, Mackness M, Aviram M, Paragh G, Eds. *The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism.* Pp 103–128. Dordrecht: Springer, 2008.

87. Boright AP, Connelly PW, Brunt JH, Scherer SW, Tsui LC, Hegele RA. Genetic variation in paraoxonase-1 and paraoxonase-2 is associated with variation in plasma lipoproteins in Alberta Hutterites. *Atherosclerosis* 1998; 139: 131–136.

88. Sanghera DK, Aston CE, Saha N, Kamboh MI. DNA polymorphisms in two paraoxonase genes (PON1 and PON2) are associated with the risk of coronary artery disease. *Am J Hum Genet* 1998; 62: 36–44.

89. Reddy ST, Wadleigh DJ, Grijalva V, Ng C, Hama S, Gangopadhyay A, Shih DM, Lusis AJ, Navab M, Fogelman AM. Human paraoxonase-3 is an HDL associated-enzyme with biological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids. *Arterioscler Thromb Vasc Biol* 2001; 21: 542–547.

90. Draganov D, Stetson PL, Watson CE, Billecke S, La Du BN. Rabbit serum paraoxonase 3 (PON3) is a high density lipoprotein-associated lactonase and protects low density lipoprotein against oxidation. *J Biol Chem* 2000; 275: 33435–33442.

91. Teiber JF, Draganov DI, La Du BN. Lactonase and lactonizing activities of human serum paraoxonase (PON1) and rabbit serum PON3. *Biochem Pharmacol* 2003; 66: 887–896.

VI *MATERIAL SUPLEMENTARIO*

Supportive/Supplementary Tables and Figures

Coordinated function of CCL2 and PON1

The role of combined assessment of defense against oxidative stress and inflammation in the evaluation of peripheral arterial disease

Anna Rull[1], Raúl García[2], Laura Fernández-Sender[1], Raúl Beltrán-Debón[1], Gerard Aragonès[1], Josep Maria Alegret [3], Carlos Alonso-Villaverde [4], Bharti Mackness[1], Michael Mackness [1], Jordi Camps[1], Vicente Martin-Paredero[2], Jorge Joven[1,*]

[1] Centre de Recerca Biomèdica, IISPV, Universitat Rovira i Virgili, Spain; [2] Servei d'Angiologia, Cirurgia Vascular i Endovascular, Hospital Universitari Joan XXIII de Tarragona, Universitat Rovira i Virgili, Spain; [3] Cardiology Unit, Hospital de Sant Joan. Reus, Spain; [4] Servei de Medicina Interna. Hospital Son Llàtzer, Palma, Mallorca, Spain.

[*] Corresponding author at: Centre de Recerca Biomèdica, IISPV, Universitat Rovira i Virgili. Carrer Sant Llorenç 21, 43201, Reus, Spain.Tel. +34977310300. Fax. +34977342105 E-mail: jorge.joven@urv.cat

Supportive/Supplementary Table I. Summary of primers and identification for the *CCL2* and *PON1* SNPs genotyped in this study.

Genomic Localization	SNP ID	Primer probes
CCL2		
-2835 C/A	rs2857654	TGTGTCCCCAAGCGAGAGTCCAACC[**C/A**]AGGTTTGTGCCAGAGCCTAACCCAG
-2578 A/G	rs1024611	GAGCAGAAGTGGGAGGCAGACAGCT[**A/G**]TCACTTTCCAGAAGACTTTCTTTTC
-2136 A/T	rs1024610	AGTTCATGGTAAAGGATGCACTAAC[**A/T**]GATTAGAGAGAGGTTTCCCCTGATA
-1811 A/G	rs3760399	AATTCCACCAGAGTCTGAAATGGCC[**A/G**]CTCCATAGAGTCTCTGCTCTGGGAT
-927 G/C	rs3760396	TGAGGACAGAGAGAGGACCCAAGCA[**G/C**]GCAACTAGTTGGAGGACTTGTACAC
+3726 T/C	rs2530797	TGCAAACAAATGAGACCTCATCATA[**T/C**]GGCTTCCGAGCAGCAACCTTTTGAC
PON1		
-1741 C/T	rs757158	TTGACATGCCCACTGGGGTGTTTAA[**C/T**]AGTCTTTTCAAAATGTGTCCAAAGG
-1076 A/G	rs854573	AACATGCTTTACTTATCACCTTTAG[**A/G**]TTTCTGGGGGGAAGCTTCTTTGGCC
-909 C/G	rs854572	AGGAGGTGCCTCTGTACAACCATGT[**C/G**]TCTCTTCTCTGCTGTCTGCTGTTAC
-832 C/T	rs854571	AGGAGGTGCCTCTGTACAACCATGT[**C/T**]TCTCTTCTCTGCTGTCTGCTGTTAC
-162 G/A	rs705381	GGGCCGACCAGGTGCACAGAAGGCG[**G/A**]GGCTTGCGGTCAGCCCCCACCGCAG
+55 L/M	rs854560	GCCAGTCCATTAGGCAGTATCTCCA[**L/M**]GTCTTCAGAGCCAGTTTCTGCCAGA
+192 Q/R	rs662	TAAACCCAAATACATCTCCCAGGAT[**Q/R**]GTAAGTAGGGGTCAAGAAAATAGTG

Genomic Localization is given in bp relative to the transcription start site (position 0).
L, Leu; M, Met; Q, Gln; R, Arg.

Supportive/Supplementary Table II. Degree of internal carotid artery (ICA) stenosis in patients with peripheral artery disease (PAD).

	Total PAD patients (n=85)		Moderate PAD patients (n=54)		Severe PAD patients (n=31)	
Arterial diameter reduction	**Right ICA**	**Left ICA**	**Right ICA**	**Left ICA**	**Right ICA**	**Left ICA**
0% (normal)	0	0	0	0	0	0
1% - 15%	17.6 (15)	15.3 (13)	22.2 (12)	20.4 (11)	9.7 (3)	6.5 (2)
16% - 49%	**67.1 (57)**	**70.6 (60)**	**66.7 (36)**	**66.7 (74)**	**77.4 (24)**	**77.4 (24)**
50% - 79%	11.8 (10)	12.9 (11)	5.6 (3)	11.1 (13)	16.1 (5)	16.1 (5)
80% - 99%	3.6 (3)	1.2 (1)	5.6 (3)	1.9 (1)	0	0

Degree of ICA stenosis was defined by the criteria of University of Washington. Values are given as percentage (n).

Supportive/Supplementary Table III. Indications for treatment in patients with peripheral artery disease (PAD) included in the study and segregated according to ABI values.

	Total PAD patients n =85	Moderate PAD n =54	Severe PAD n =31	p-value
Elective Treatment, %				
Medication	23.2	**32.7**	**13.8**	**0.063**
Endovascular treatment	12.6	13.5	10.3	0.683
Superficial femoral	*6.3*	*5.8*	*6.9*	*0.840*
Distal segment	*2.1*	*1.9*	*0.0*	*0.452*
Iliac artery	*4.2*	*5.8*	*3.4*	*0.644*
Stenting	7.4	9.6	3.4	0.320
Bypass surgery	34.7	**26.9**	**55.2**	**0.012**
Femoro-femoral/popliteal/tibial	*23.2*	*21.2*	*27.6*	*0.512*
Aorto-bifemoral	*6.3*	*3.8*	*13.8*	*0.101*
Axillo-bifemoral	*5.3*	***1.9***	***13.8***	***0.033***
Amputation	22.1	17.3	17.2	0.994

Moderate PAD defined by ABI between 0.40-0.90. Severe PAD defined by ABI <0.40.

UNIVERSITAT ROVIRA I VIRGILI
INFLAMACIÓN Y ESTRÉS OXIDATIVO EN EL SÍNDROME DE ISQUEMIA CRÓNICA DE MIEMBROS INFERIORES
Raúl Garcia Vidal
Dipòsit Legal: T. 191-2013

Supportive/Supplementary Table IV. Correlation matrix of inflammatory and PON1-related variables measured in patients with peripheral artery disease.

	β2M	CCL2	hsCRP	IL-6	IL-8	Lactonase	PONa	PON1	sCD40L	sP-Selectin	t-PA
β2M		**r = 0.368 p<0.001**	**r = 0.407 p<0.001**	r = 0.237 p= 0.081	r = -0.295 p= 0.096	**r = -0.339 p= 0.002**	**r = -0.422 p<0.001**	**r = 0.305 p= 0.005**	r = 0.116 p= 0.300	r = -0.008 p= 0.943	r =- 0.124 p= 0.352
CCL2			r = 0.148 p= 0.179	r = 0.019 p= 0.879	r = 0.231 p= 0.163	**r = -0.293 p= 0.003**	**r = -0.331 p<0.001**	r = -0.056 p= 0.586	r = 0.174 p= 0.087	r = 0.141 p= 0.165	r = -0.052 p= 0.610
hsCRP				**r = 0.595 p<0.001**	r = 0.202 p= 0.244	**r = -0.339 p= 0.002**	**r = -0.394 p<0.001**	r = 0.185 p= 0.091	r = 0.106 p= 0.339	r = -0.008 p= 0.943	r = -0.183 p= 0.096
IL-6					r = 0.241 p= 0.208	r = -0.200 p= 0.104	r = -0.209 p= 0.090	r = -0.184 p= 0.137	r =0.065 p= 0.600	r = 0.119 p= 0.336	r = -0.059 p= 0.637
IL-8						r = 0.016 p= 0.923	r = 0.196 p= 0.237	r = 0.021 p= 0.903	r = -0.036 p= 0.830	r = 0.081 p= 0.628	r = -0.093 p= 0.579
Lactonase							**r = 0.591 p<0.001**	r = -0.016 p= 0.115	r = -0.165 p= 0.105	**r = -0.231 p= 0.022**	r = 0.078 p= 0.443
PONa								r = -0.084 p= 0.412	r = 0.033 p= 0.748	r = 0.009 p= 0.930	r = 0.091 p= 0.377
PON1									r = -0.004 p= 0.968	r = -0.106 p= 0.302	r = 0.127 p= 0.215
sCD40L										**r = 0.363 p<0.001**	r = -0.094 p= 0.360
sP-Selectin											r = -0.036 p= 0.728
t-PA											

Supportive/Supplementary Table V. Genotype frequencies and statistical assessment of comparisons between controls and affected patients (PAD) with co-dominant, dominant and recessive models of the selected CCL2 single nucleotide polymorphisms.

		Control	PAD	Co-dominant		Dominant		Recessive	
Gene	**Genotype**	*%*	*%*	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
-2835 C/A	CC	57	58	1.00 (reference)	0.92	1.00 (reference)	0.80	1.00 (reference)	0.81
rs2857654	CA	37	35	1.08 (0.71-1.64)		1.05 (0.71-1.56)			
	AA	6	7	0.93 (0.42-2.05)				0.91 (0.42-1.96)	
-2578 A/G	AA	57	58	1.00 (reference)	0.98	1.00 (reference)	0.87	1.00 (reference)	0.97
rs1024611	AG	37	36	1.04 (0.68-1.58)		1.04 (0.69-1.54)			
	GG	6	6	1.00 (0.43-2.30)				0.98 (0.43-2.22)	
-2136 A/T	AA	62	64	1.00 (reference)	0.94	1.00 (reference)	0.73	1.00 (reference)	0.93
rs1024610	AT	32	31	1.08 (0.70-1.66)		1.07 (0.71-1.62)			
	TT	6	5	1.07 (0.44-2.59)				1.04 (0.43-2.50)	
-1811 A/G	AA	89	87	1.00 (reference)	0.45	not applied due to the absence of GG individuals			
rs3760399	AG	11	13	1.79 (0.44-1.42)					
	GG	-	-						
-927 G/C	GG	71	73	1.00 (reference)	0.40	1.00 (reference)	0.70	1.00 (reference)	0.25
rs3760396	CG	27	24	1.18 (0.74-1.87)		1.09 (0.70-1.69)			
	CC	11	4	0.51 (0.16-1.59)				0.49 (0.16-1.52)	
+3726 T/C	TT	33	35	1.00 (reference)	0.88	1.00 (reference)	0.72	1.00 (reference)	0.64
rs2530797	TC	47	47	1.05 (0.67-1.63)		1.13 (0.67-1.89)			
	CC	20	18	1.16 (0.65-2.06)				0.99 (0.67-1.48)	

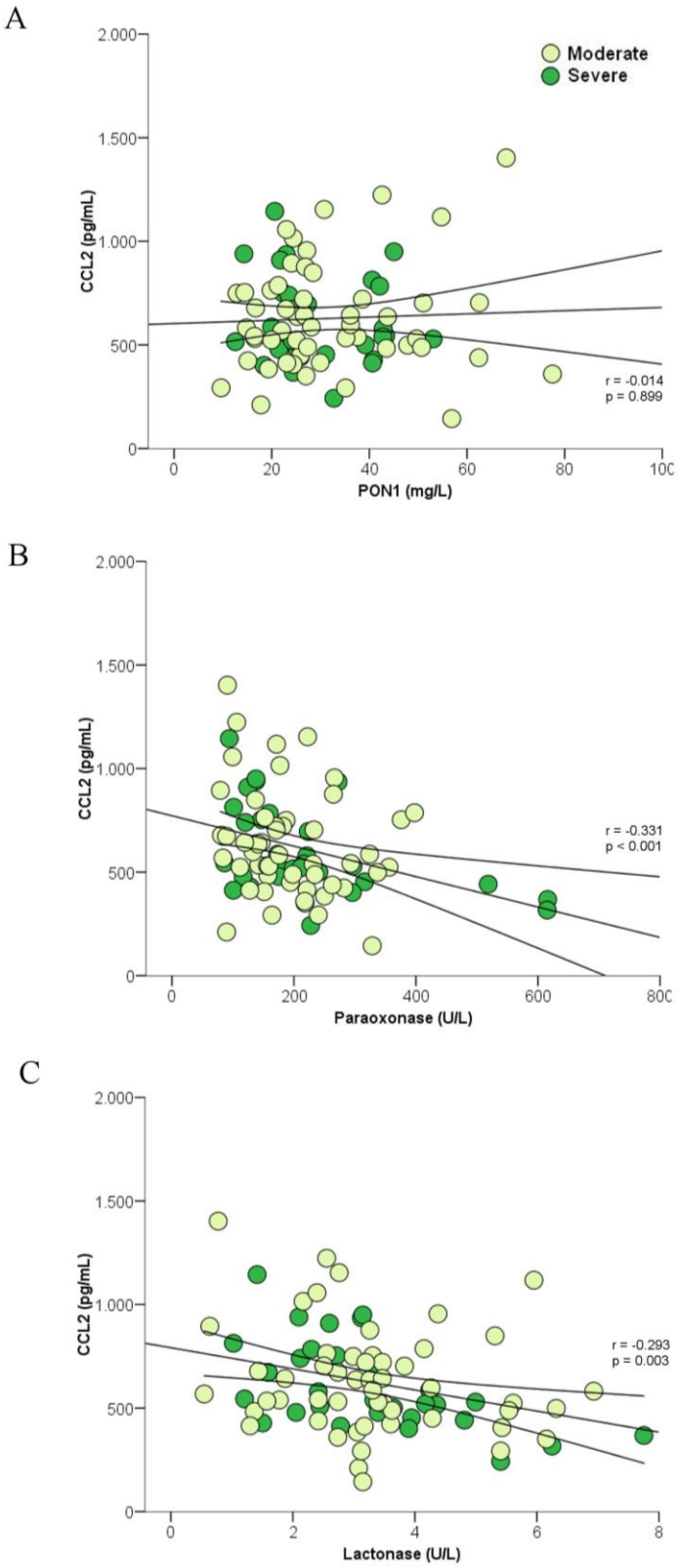
OR: odds ratio; CI: confidence interval

Supportive/Supplementary Table VI. Genotype frequencies and statistical assessment of comparisons between controls and affected patients (PAD) with co-dominant, dominant and recessive models of the selected PON1 single nucleotide polymorphisms.

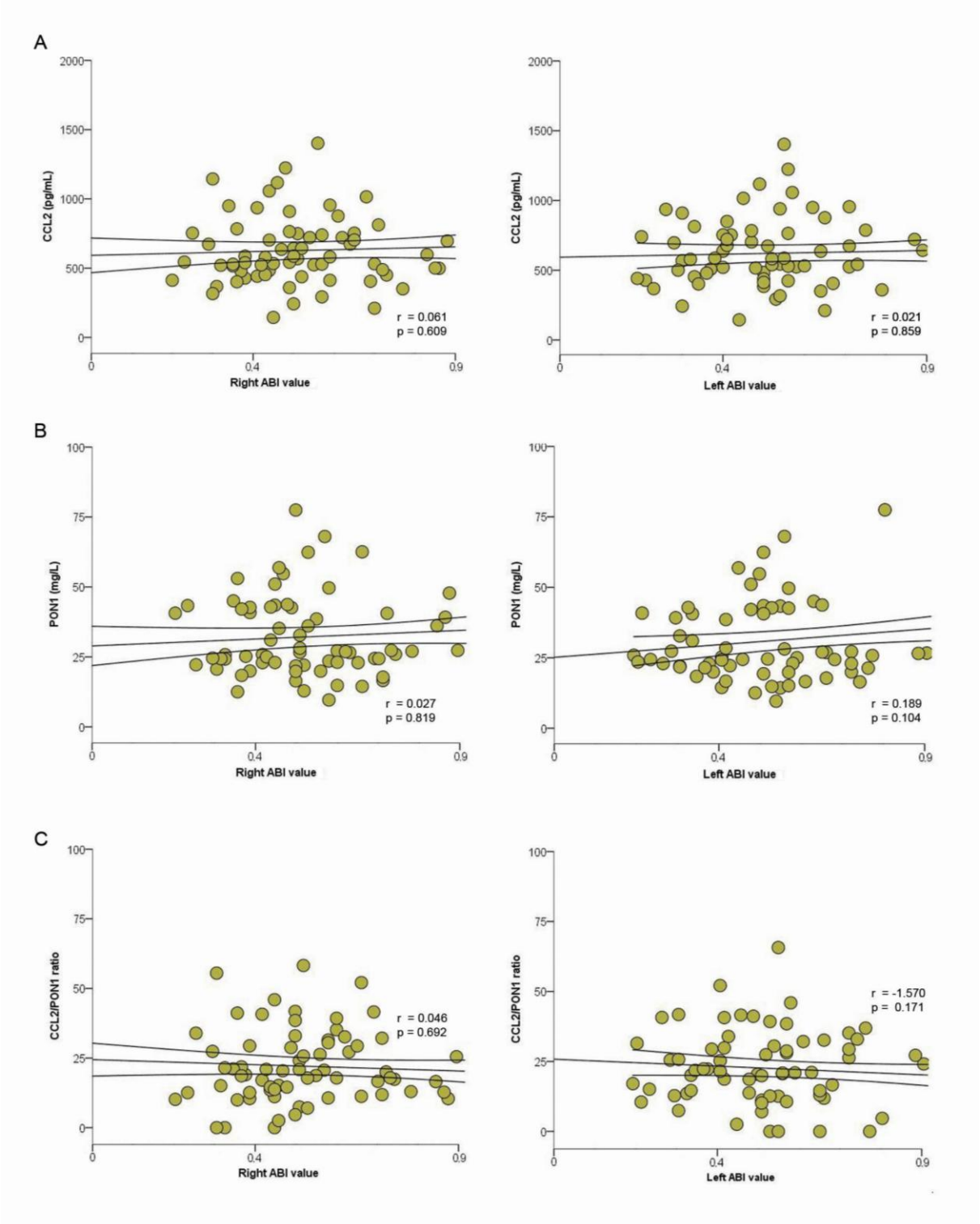
		Control	PAD	Co-dominant		Dominant		Recessive	
Gene	**Genotype**	*%*	*%*	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**	**OR (95% CI)**	**p-value**
-1741 C/T	CC	40	41	1.00 (reference)		1.00 (reference)		1.00 (reference)	
rs757158	CT	46	47	1.01 (0.66-1.53)	0.71	1.06 (0.71-1.58)	0.77		0.41
	TT	14	11	1.29 (0.67-2.47)				1.28 (0.70-2.36)	
-1076 A/G	AA	60	58	1.00 (reference)		1.00 (reference)		1.00 (reference)	
rs854573	AG	35	37	0.92 (0.61-1.40)	0.82	0.90 (0.61-1.35)	0.62		0.61
	GG	4	5	0.76 (0.31-1.89)				0.79 (0.32-1.92)	
-909 C/G	CC	37	37	1.00 (reference)		1.00 (reference)		1.00 (reference)	
rs854572	CG	46	47	0.98 (0.63-1.51)	0.96	1.00 (0.66-1.51)	1.00		0.79
	GG	17	16	1.06 (0.59-1.91)				1.07 (0.63-1.84)	
-832 C/T	CC	54	54	1.00 (reference)		1.00 (reference)		1.00 (reference)	
rs854571	CT	40	39	1.04 (0.69-1.58)	0.85	1.01 (0.68-1.50)	0.96		0.59
	TT	6	7	0.82 (0.37-1.81)				0.80 (0.37-1.75)	
-162 G/A	GG	61	58	1.00 (reference)		1.00 (reference)		1.00 (reference)	
rs705381	GA	35	37	0.92 (0.60-1.39)	0.79	0.90 (0.60-1.34)	0.59		0.58
	AA	4	6	0.75 (0.30-1.85)				0.77 (0.32-1.88)	
+55 L/M	LL	37	33	1.00 (reference)		1.00 (reference)		1.00 (reference)	
rs854560	LM	47	46	0.91 (0.59-1.43)	0.38	0.84 (0.55-1.27)	0.40		0.18
	MM	16	21	0.67 (0.39-1.17)				0.71 (0.44-1.16)	
+192 Q/R	QQ	48	54	1.00 (reference)		1.00 (reference)		1.00 (reference)	
rs662	QR	43	39	1.25 (0.83-1.89)	0.49	1.27 (0.85-1.88)	0.24		0.61
	RR	8	7	1.34 (0.61-2.93)				1.21 (0.57-2.60)	

OR: odds ratio; CI: confidence interval

Supportive/Supplementary Figure I. Individual plasma CCL2 concentration plotted against the corresponding PON1 concentration (A), paraoxonase activity (B) and lactonase activity (C) in PAD patients.

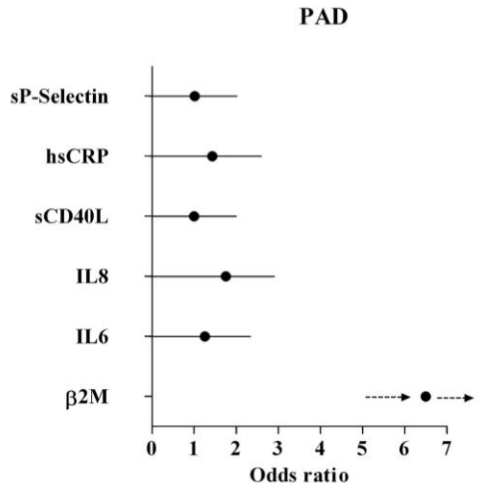


Supportive/Supplementary Figure II. Individual plasma CCL2 concentration (A), serum PON1 levels (B), and CCL2/PON1 ratio (C) plotted against the corresponding ABI values in PAD patients.



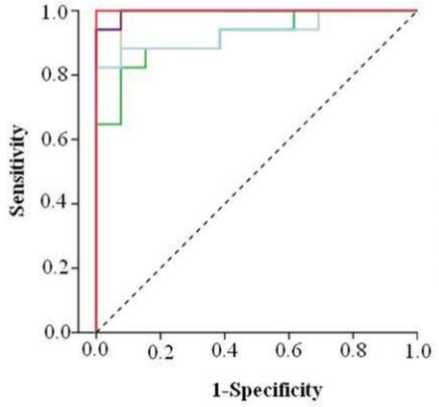
Supportive/Supplementary Figure III. Diagnosis of PAD based on plasma biomarkers (A). Receiving operating characteristic curves and areas under the curve (AUC) values for predicting PAD with individual (B) and combined biomarkers (C). Adjusted model includes BMI and smoking.

A



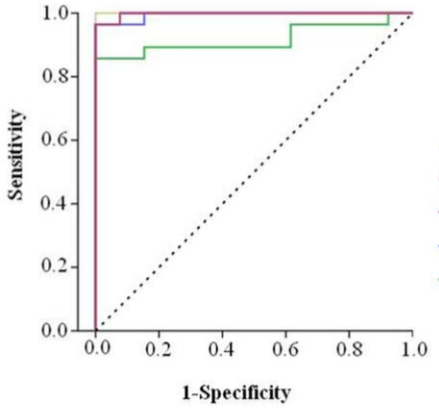
	OR	95% CI	adjusted p-value
sP-Selectin	1.014	1.002- 1.025	**0.02**
hsCRP	1.429	1.161-1.759	**0.02**
sCD40L	1.000	1.000-1.000	**0.02**
IL8	1.754	1.150-2.676	**0.01**
IL6	1.253	1.081-1.453	**0.01**
β2M	> 10	1.700->10	**0.05**

B



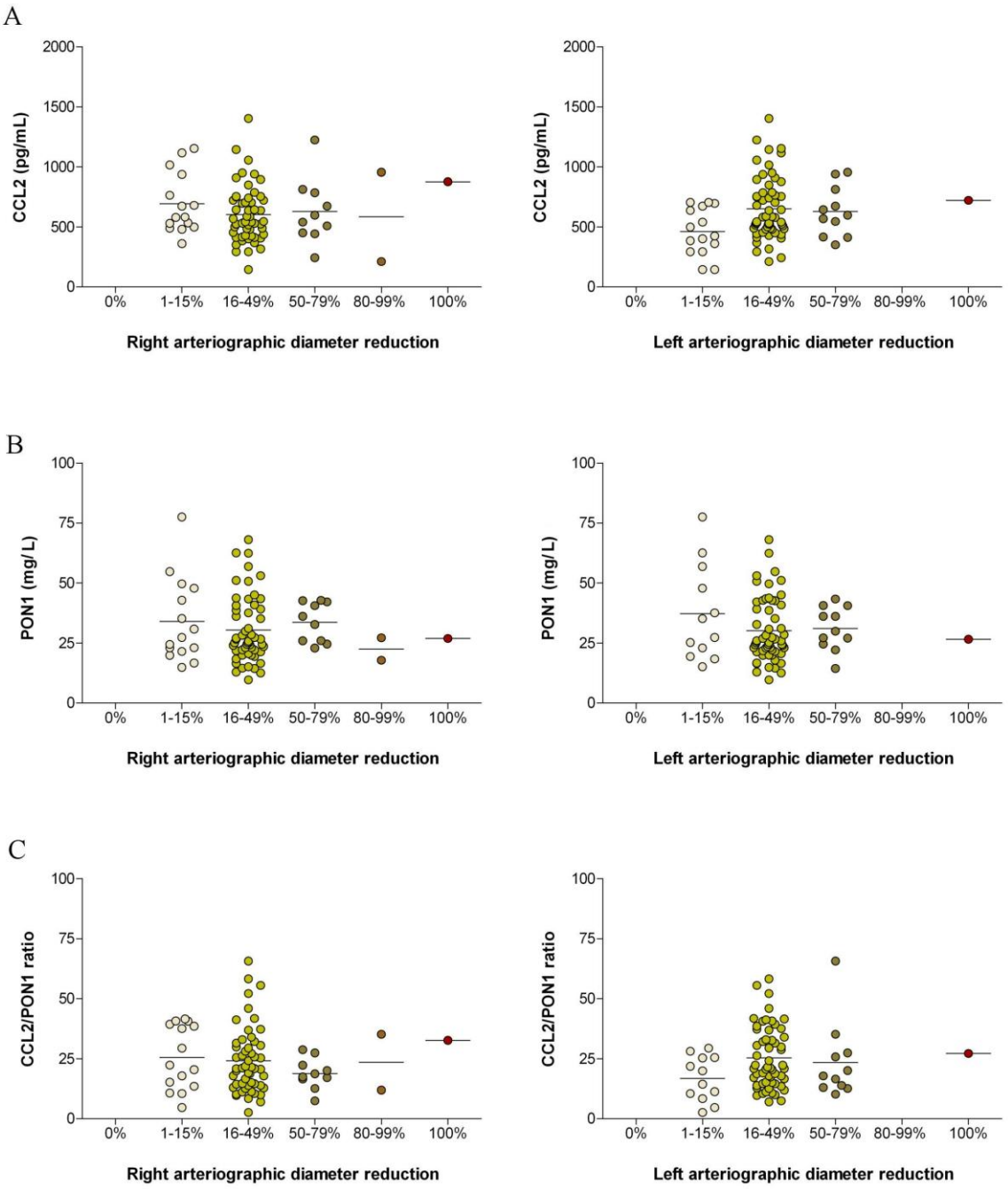
	AUC	95% CI
β2M	1.000 ± 0.000	1.000 - 1.000
hsCRP	0.995 ± 0.007	0.981 - 1.010
IL6	0.995 ± 0.007	0.981 - 1.010
IL8	0.986 ± 0.016	0.954 - 1.018
sCD40L	0.919 ± 0.050	0.821 - 1.016
sP-Selectin	0.932 ± 0.048	0.838 - 1.026

C

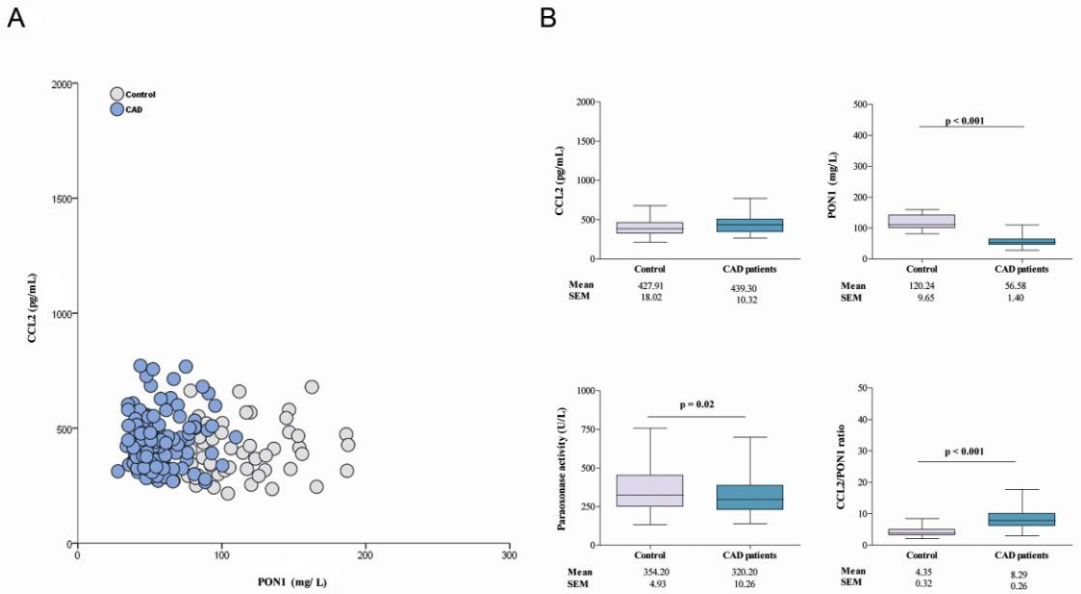


	AUC	95% CI
hsCRP + IL6	1.000 ± 0.000	1.000 - 1.000
hsCRP + sCD40L	0.997 ± 0.005	0.988 - 1.006
hsCRP + sP-Selectin	0.997 ± 0.005	0.988 - 1.006
IL8 + Il6	0.995 ± 0.007	0.980 - 1.009
sCD40L + sP-Selectin	0.997 ± 0.005	0.988 - 1.006

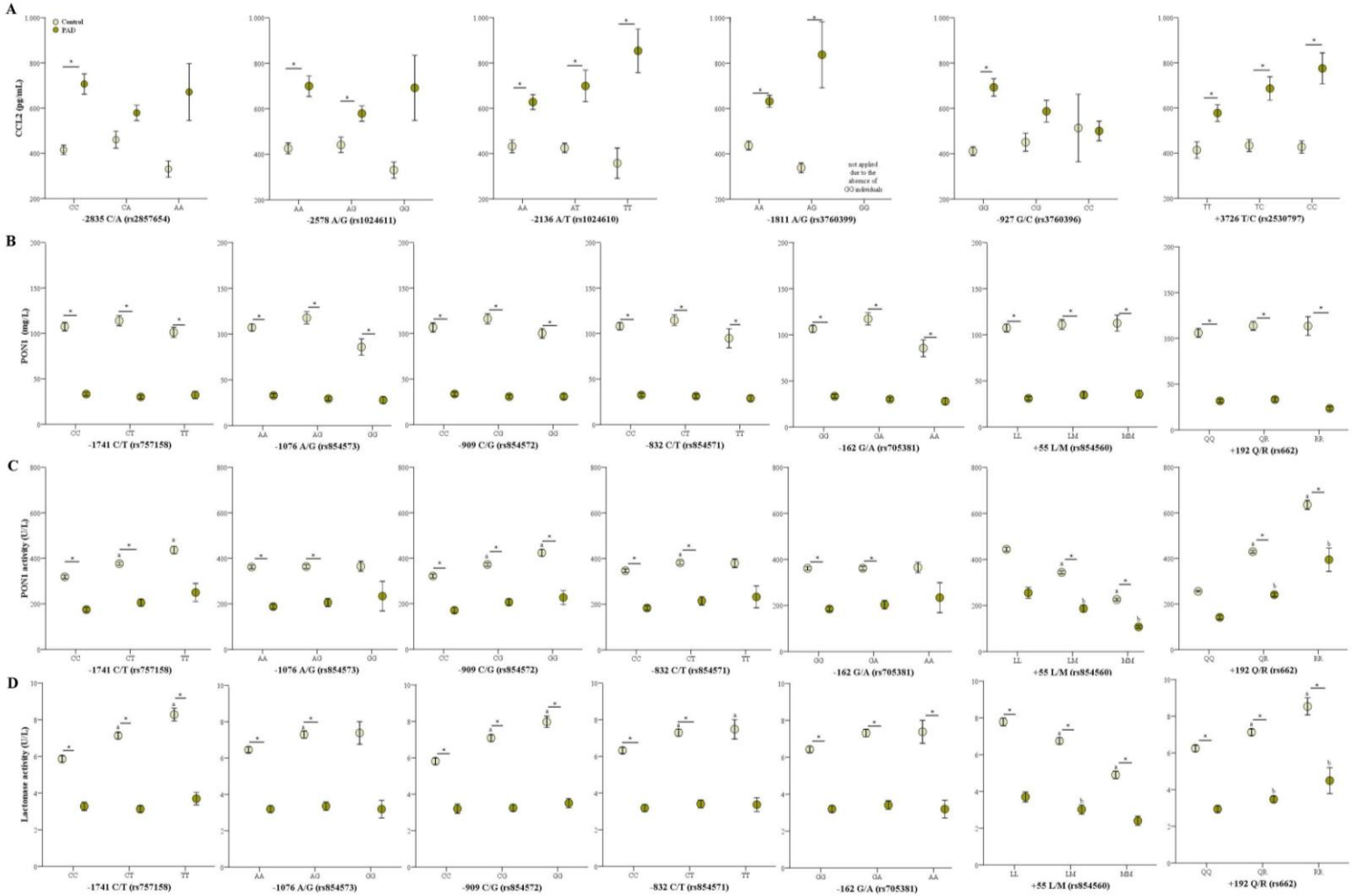
Supportive/Supplementary Figure IV. Plasma CCL2 concentration, serum PON1 levels and CCL2/PON1 ratio in PAD patients according to the degree of internal carotid artery (ICA) stenosis.


A
CCL2 (pg/mL)
2000
1500
1000
500
0
0%
1-15%
16-49%
50-79%
80-99%
100%
Right arteriographic diameter reduction
Left arteriographic diameter reduction
B
PON1 (mg/L)
100
75
50
25
0
C
CCL2/PON1 ratio

Supportive/Supplementary Figure V. Plasma CCL2 values, serum PON1 concentration and activity, and CCL2/PON1 ratio in survivors of myocardial infarction (CAD, coronary artery disease) as compared with unaffected controls.



Supportive/Supplementary Figure VI. Effect of *CCL2* genotype variants in plasma CCL2 concentration (A) and that of *PON1* genotype variants in serum PON1 concentration (B), paraoxonase activity (C) and lactonase activity (D). *p<0.05 in PAD patients compared to healthy patients. [a]p<0.05 Difference in genetic variants compared to the most common genotype in each SNPs from the same group. [b]p<0.05 Difference in genetic variants compared to the most common genotype in each SNPs from PAD patients.



SUPPLEMENTARY DATA

Serum paraoxonase-3 concentration is associated with insulin sensitivity in peripheral artery disease and with inflammation in coronary artery disease

Supplementary Table 1

Degree of internal carotid artery (ICA) stenosis defined by the criteria of University of Washington in patients with peripheral artery disease (PAD).

	Total PAD patients (n=118)	
Arterial diameter reduction	Right ICA	Left ICA
0% (normal)	0	0
1% - 15%	19.5 (23)	17.8 (21)
16% - 49%	61.9 (73)	67.4 (76)
50% - 79%	14.4 (17)	16.4 (20)
80% - 99%	3.8 (4)	-
Occlusion (100%)	0.8 (1)	0.8 (1)

Values are given as percentage (n).

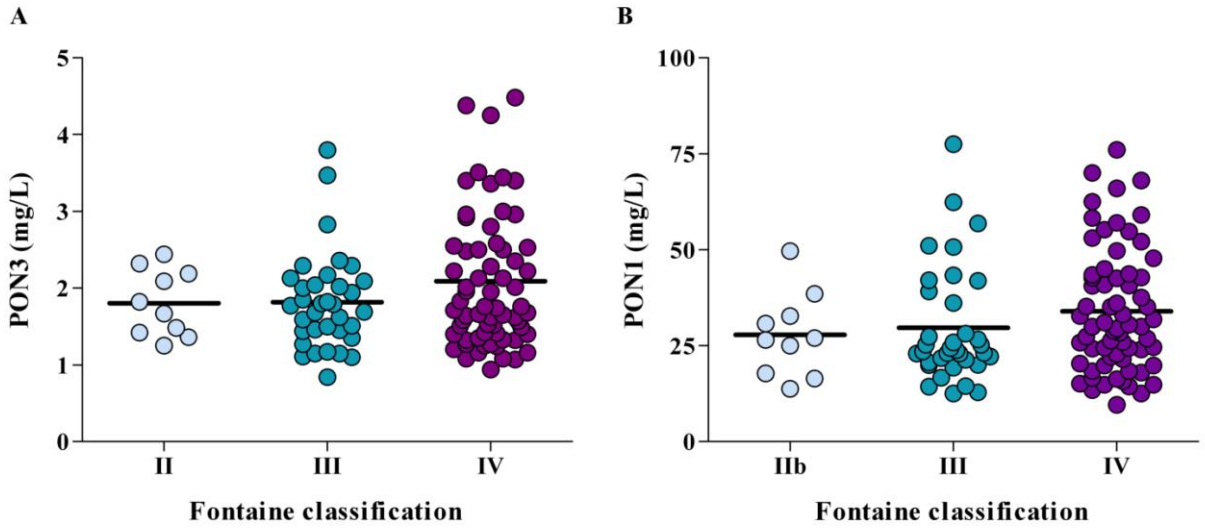
Supplementary Table 2

Correlation coefficients of the regression lines between serum PON3 concentrations and PON1-related measurements in patients with coronary artery disease (CAD) and peripheral artery disease (PAD).

	CAD patients (n =72)		PAD patients (n =118)	
Parameter	Spearman's ρ	*P-value*	Spearman's ρ	*P*-value
PON1 concentration	0.576	1.204E-07	0.521	4.640E-09
Paraoxonase activity	0.102	0.398	-0.024	0.804
Lactonase activity	-0.081	0.503	-0.058	0.544

Supplementary Figure 1

Serum PON3 concentration in patients with peripheral artery disease classified by Fontaine index.
Fontaine classification was defined as IIb, moderate to severe claudication; III, ischemic rest pain; and IV, ulceration or gangrene.



Supplementary Table 3

Clinical characteristics and biochemical variables of patients with peripheral artery disease (PAD) classified by Fontaine index

	Total PAD patients (n =118)	PAD patients by Fontaine classification			*P*-value
		IIb	III	IV	
Clinical characteristics, %					
Age, years	70.65 (0.79)	68.60 (2.72)	67.30 (1.52)	72.66 (0.93)	0.010
Gender, male	85.6 %	100 %	94.4 %	78.9 %	0.039
BMI, Kg/m^2	24.91 (0.30)	25.60 (0.99)	25.88 (0.57)	24.32 (0.36)	0.076
Biochemical variables					
Total cholesterol, mmol/L	3.98 (0.09)	4.23 (0.26)	3.89 (0.17)	3.99 (0.12)	0.582
HDL-cholesterol, mmol/L	1.08 (0.03)	0.82 (0.08)	1.08 (0.05)	1.10 (0.04)	0.065
LDL-cholesterol, mmol/L	2.30 (0.09)	2.60 (0.31)	2.27 (0.14)	2.28 (0.12)	0.600
Triglycerides, mmol/L	2.98 (0.10)	3.18 (0.16)	3.04 (0.18)	2.89 (0.13)	0.582
Glucose, mmol/L	6.55 (0.25)	6.36 (0.50)	6.19 (0.36)	6.76 (0.36)	0.614
Insulin, pmol/L	114.71 (11.17)	156.21 (46.94)	99.52 (21.87)	110.45 (12.18)	0.240
HOMA-IR	2.19 (0.20)	2.81 (0.76)	1.84 (0.38)	2.18 (0.24)	0.188
PON-related variables					
PON1 concentration, mg/L	32.02 (1.43)	27.32 (3.43)	29.69 (2.55)	33.90 (1.93)	0.292
PON3 concentration, mg/L	1.98 (0.08)	1.80 (0.13)	1.81 (0.10)	2.08 (1.11)	0.602
Lactonase activity, U/L	3.31 (0.14)	3.65 (0.40)	3.10 (0.17)	3.34 (0.20)	0.575
Paraoxonase activity, U/L	197.94 (9.66)	201.18 (24.04)	192.29 (17.10)	199.34 (13.20)	0.769
Inflammation-related markers					
β2M, mg/L	4.15 (0.28)	3.45 (0.33)	4.10 (0.59)	4.39 (0.36)	0.265
CCL2, ng/L	656.33 (320.33)	620.38 (82.94)	633.18 (36.56)	669.13 (45.06)	0.899
hs-CRP, mg/L	58.16 (7.33)	26.66 (10.05)	51.69 (13.00)	68.32 (10.29)	0.210

Values are given as mean ± SEM or percentages.
Fontaine classification was defined as IIb, moderate to severe claudication; III, ischemic rest pain; and IV, ulceration or gangrene.

β2M: β-2-microglobulin; hs-CRP: high-sensitivity C-reactive protein.

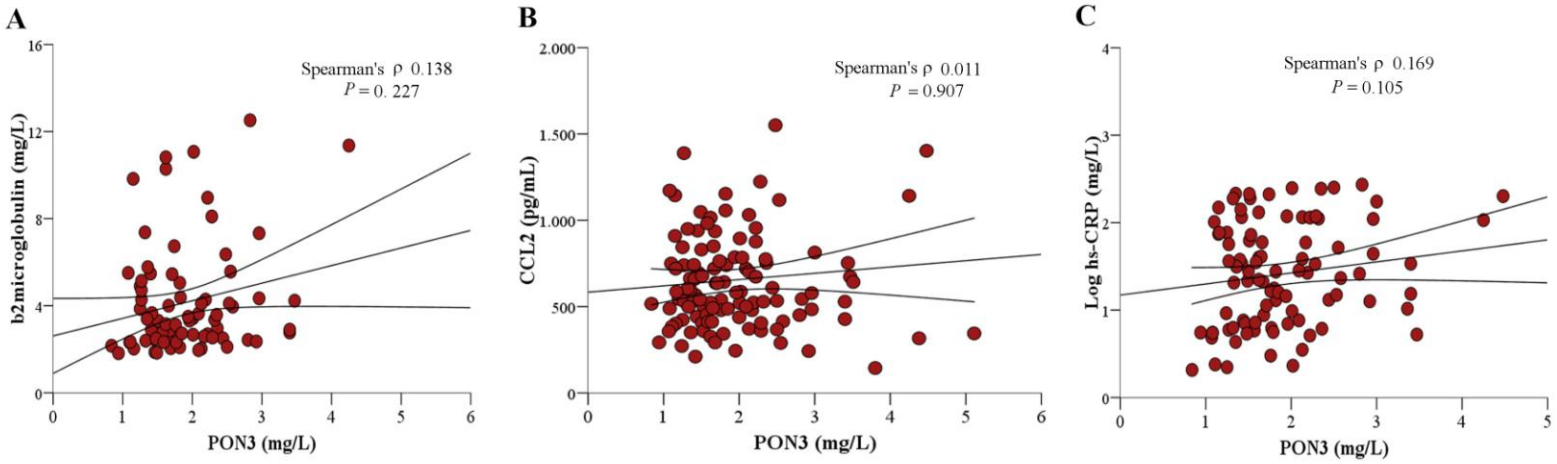
Supplementary Table 4

Multiple regression analysis of the determinants of PON3 concentration in patients with peripheral artery disease (PAD).

	Unstandardized coefficients		Standardized coefficients		
	B	Std. Error	Beta	t	*P*-value
Gender	-0.098	0.277	-0.046	-0.353	0.725
BMI, kg/m^2	0.037	0.031	0.145	1.200	0.234
Total cholesterol, mmol/L	0.274	0.298	0.335	0.918	0.362
HDL-cholesterol, mmol/L	-0.468	0.437	-0.189	-1.071	0.288
LDL-cholesterol, mmol/L	-0.333	0.283	-0.362	-1.177	0.243
Triglycerides, mmol/L	-0.215	0.115	-0.290	-1.868	0.066
Glucose, mmol/L	-0.031	0.034	-0.110	-0.916	0.363
Insulin, pmol/L	0.002	0.001	0.321	2.667	0.010
Diabetes diagnosis	0.259	0.218	0.148	1.184	0.240

BMI, body mass index.

Supplementary Figure 2
Relationships between serum PON3 concentrations and inflammation markers in patients with peripheral artery disease (PAD)


A
Spearman's ρ 0.138
P = 0. 227
b2microglobulin (mg/L)
PON3 (mg/L)
B
Spearman's ρ 0.011
P = 0.907
CCL2 (pg/mL)
PON3 (mg/L)
C
Spearman's ρ 0.169
P = 0.105
Log hs-CRP (mg/L)
PON3 (mg/L)

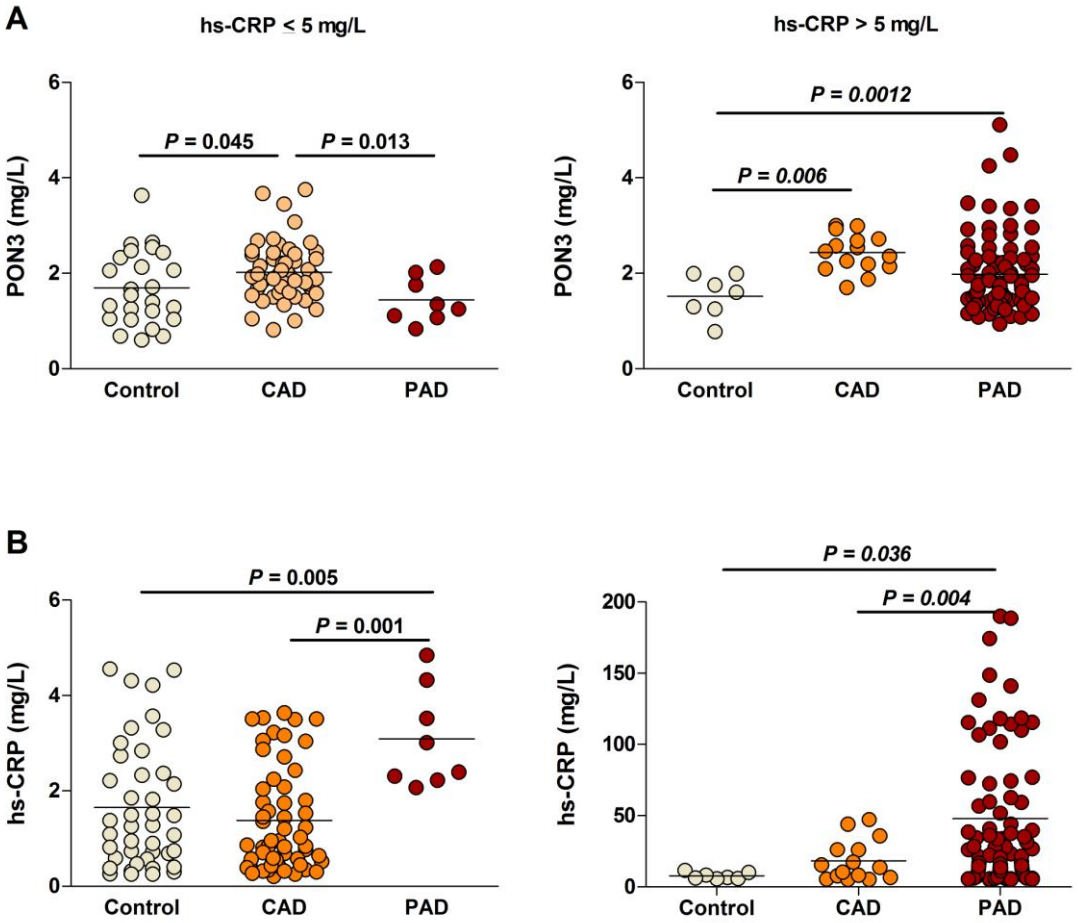
Supplementary Table 5

Multiple regression analysis of the determinants of PON3 concentration in patients with coronary artery disease (CAD).

	Unstandardized coefficients		Standardized coefficients		
	B	Std. Error	Beta	t	*P*-value
HDL-cholesterol, mmol/L	0.096	0.103	0.126	0.929	0.358
LDL-cholesterol, mmol/L	0.131	0.072	0.257	1.805	0.077
Triglycerides, mmol/L	-0.104	0.076	-0.224	-1.366	0.178
Glucose, mmol/L	0.051	0.039	0.205	1.314	0.195
Insulin, pmol/L	0.000	0.002	-0.019	-0.112	0.911
CCL2, ng/L	0.001	0.001	0.160	1.217	0.230
β2m, mg/L	0.287	0.141	0.299	2.038	0.047
hs-CRP, mg/L	0.010	0.008	0.169	1.273	0.209

Supplementary Figure 3

Serum PON3 concentration in control subjects and in patients with coronary artery disease (CAD) or peripheral artery disease (CAD), and segregated according to their CRP levels.



Supplementary Table 6

Genotype frequencies and statistical assessment of comparisons between controls and CAD patients with co-dominant, dominant and recessive models of the selected PON3 single nucleotide polymorphisms

Gene	Genotype	CAD *n (%)*	Control *n (%)*	Co-dominant		Dominant		Recessive	
				OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
-4984 A/G	AA	45 (66.2)	87 (58.8)	1.00 (reference)		1.00 (reference)			
-	AG	21 (30.9)	56 (37.8)	0.725 (0.391 - 1.344)	0.584	0.729 (0.400 - 1.328)	0.301	1.00 (reference)	0.798
	GG	2 (2.9)	5 (3.4)	0.773 (0.144 - 4.144)				0.866 (0.164 - 4.583)	
-4970 T/G	TT	46 (65.7)	87 (58.8)	1.00 (reference)		1.00 (reference)			
-	TG	22 (31.4)	56 (37.8)	0.743 (0.404 - 1.366)	0.619	0.744 (0.411 - 1.345)	0.327	1.00 (reference)	0.838
	GG	2 (2.9)	5 (3.4)	0.756 (0.141 - 4.052)				0.841 (0.159 - 4.446)	
-4105 G/A	GG	46 (65.7)	85 (57.4)	1.00 (reference)		1.00 (reference)			
-	GA	22 (31.4)	58 (39.2)	0.701(0.382 - 1.287)	0.506	0.704 (0.389 - 1.271)	0.243	1.00 (reference)	0.838
	AA	2 (2.9)	5 (3.4)	0.739 (0.138 - 3.960)				0.841 (0.159 - 4.446)	
-746 C/T	CC	43 (61.4)	83 (56.5)	1.00 (reference)		1.00 (reference)			
rs17882539	CT	24 (34.3)	58 (39.5)	0.799 (0.437 - 1.457)	0.763	0.814 (0.455 - 1.456)	0.488	1.00 (reference)	0.943
	TT	3 (4.3)	6 (4.1)	0.965 (0.230 - 4.049)				1.052 (0.255 - 4.336)	
-665 A/G	AA	43 (61.4)	83 (56.1)	1.00 (reference)		1.00 (reference)			
rs11770903	AG	24 (34.3)	59 (39.9)	0.785 (0.430 - 1.431)	0.730	0.802 (0.448 - 1.432)	0.455	1.00 (reference	0.936
	GG	3 (4.3)	6 (4.1)	0.965 (0.230 - 4.049)				1.059 (0.257 - 4.367)	
-567 C/T	CC	43 (61.4)	83 (56.5)	1.00 (reference)		1.00 (reference)			
rs11764079	CT	24 (34.3)	58 (39.5)	0.799 (0.437 - 1.457)	0.763	0.814 (0.455 - 1.456)	0.488	1.00 (reference)	0.943
	TT	3 (4.3)	6 (4.1)	0.965 (0.230 - 4.049)				1.052 (0.255 - 4.336)	

OR: odds ratio; CI: confidence interval

Supplementary Table 7
Genotype frequencies and statistical assessment of comparisons between controls and PAD patients with co-dominant, dominant and recessive models of the selected PON3 single nucleotide polymorphisms

Gene	Genotype	PAD *n (%)*	Control *n (%)*	Co-dominant		Dominant		Recessive	
				OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
-4984 A/G	AA	70 (64.2)	87 (58.8)	1.00 (reference)		1.00 (reference)			
-	AG	36 (33.0)	56 (37.8)	0.799 (0.473 - 1.349)	0.674	0.794 (0.476 - 1.324)	0.377	1.00 (reference)	0.775
	GG	3 (2.8)	5 (3.4)	0.745 (0.172 - 3.229)				0.809 (0.189 - 3.461)	
-4970 T/G	TT	72 (65.5)	87 (58.8)	1.00 (reference)		1.00 (reference)			
-	TG	36 (32.7)	56 (37.8)	0.776 (0.461 - 1.309)	0.476	0.752 (0.451 - 1.255)	0.276	1.00 (reference)	0.445
	GG	2 (1.8)	5 (3.4)	0.483 (0.091 - 2.566)				0.529 (0.101 - 2.782)	
-4105 G/A	GG	72 (65.5)	85 (57.4)	1.00 (reference)		1.00 (reference)			
-	GA	36 (32.7)	58 (39.2)	0.732 (0.435 - 1.234)	0.376	0.712 (0.427 - 1.186)	0.192	1.00 (reference)	0.445
	AA	2 (1.8)	5 (3.4)	0.472 (0.089 - 2.507)				0.529 (0.101 - 2.782)	
-746 C/T	CC	69 (63.3)	83 (56.6)	1.00 (reference)		1.00 (reference)			
rs17882539	CT	37 (33.9)	58 (39.5)	0.775 (0.461 - 1.300)	0.517	0.752 (0.452 - 1.249)	0.270	1.00 (reference)	0.568
	TT	3 (2.8)	6 (4.1)	0.601 (0.145 - 2.494)				0.665 (0.162 - 2.720)	
-665 A/G	AA	69 (62.7)	69 (62.7)	1.00 (reference)		1.00 (reference)			
rs11770903	AG	38 (34.5)	38 (34.5)	0.775 (0.601 - 0.145)	0.531	0.758 (0.458 - 1.257)	0.283	1.00 (reference)	0.566
	GG	3 (2.7)	3 (2.7)	0.601 (0.145 - 2.494)				0.663 (0.162 - 2.713)	
-567 C/T	CC	69 (62.7)	83 (56.5)	1.00 (reference)		1.00 (reference)			
rs11764079	CT	38 (34.5)	58 (39.5)	0.788 (0.469 - 1.324)	0.562	0.770 (0.465 - 1.278)	0.312	1.00 (reference)	0.559
	TT	3 (2.7)	6 (4.1)	0.601 (0.145 - 2.494)				0.658 (0.161 - 2.694)	

OR: odds ratio; CI: confidence interval