BIOMARCADORES RELACIONADOS CON EL ESTRÉS OXIDATIVO EN LA ENFERMEDAD ARTERIAL PERIFÉRICA

Isabel Fort Gallifa

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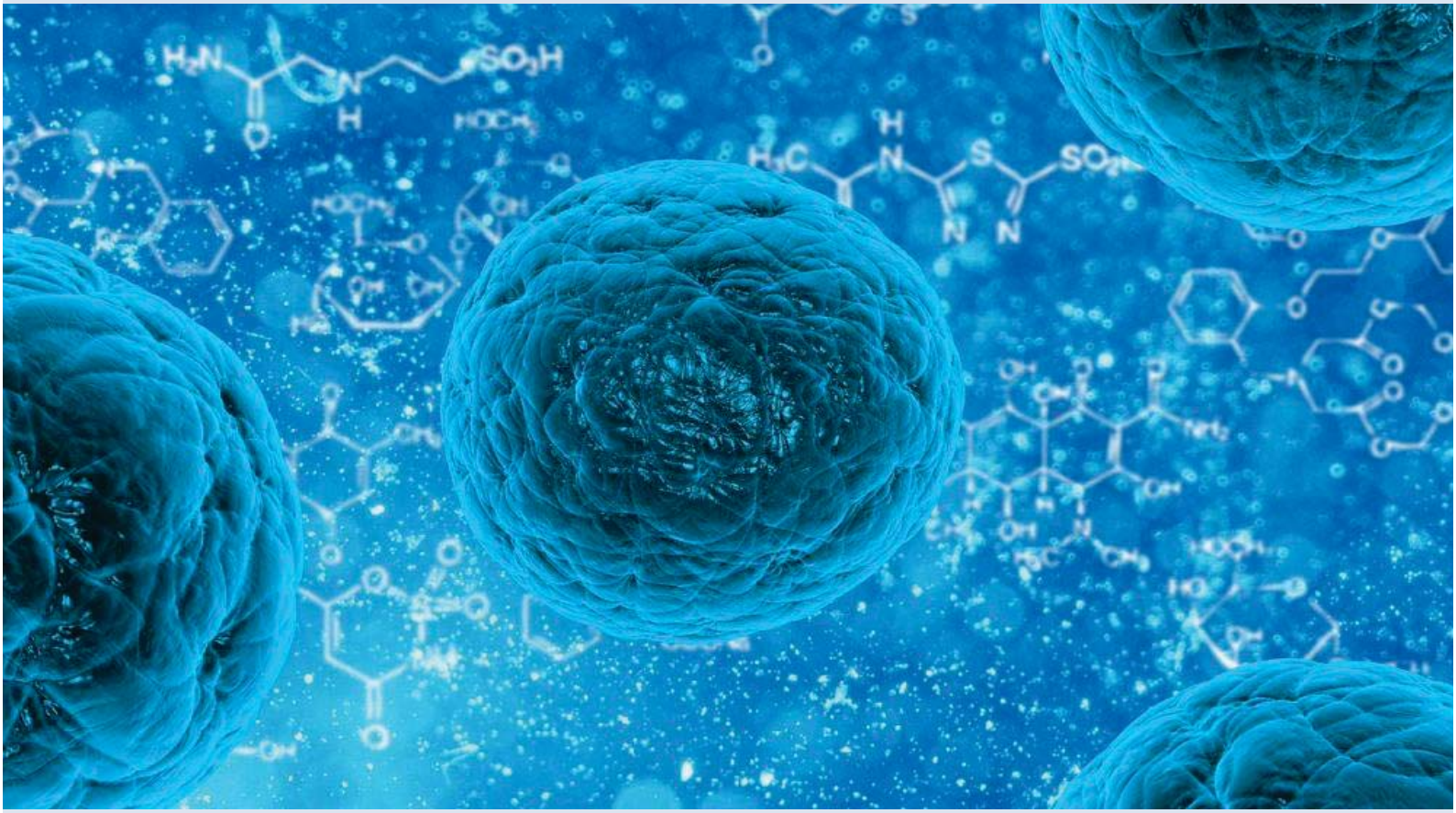

Isabel Fort Gallifa

TESIS DOCTORAL

2017

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Tesis Doctoral

Dirigida por

Dr. Jorge Camps Andreu

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Departamento de Medicina y Cirugía

UNIVERSITAT ROVIRA i VIRGILI

Reus

2017

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HAGO CONSTAR que el presente estudio, titulado "**Biomarcadores relacionados con el estrés oxidativo en la enfermedad arterial periférica**", presentado por Isabel Fort Gallifa para obtener el grado de Doctora, ha sido llevado a cabo bajo mi supervisión en el **Departamento de Medicina y Cirugía** de esta Universidad.

Reus, 27 de abril de 2017

Directores de la Tesis Doctoral

Dr. Jorge Camps Andreu

Prof. Jorge Joven Maried

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"La ciencia nunca resuelve un problema sin crear otros diez más"

George Bernard Shaw

"A doctor is a student until his/her death, when he/she fails to be a student, he/she dies"

Sir William Osler

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A mi familia

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Estos principios son los que mis padres se preocuparon por inculcarme desde pequeña, tanto a mí como a mi queridísimo hermano. Dos caracteres diferentes, la dulzura de mi madre y su capacidad para mantener la mente fría y tirar hacia adelante a pesar de las piedras que pone la vida en el camino, y la perseverancia de mi padre de una fuerza descomunal que realmente bajaría la luna por aquello que necesitase su familia. Dulzura y terquedad quedan plasmadas en esta frase que tantas veces hemos comentado en comidas o cenas familiares y con la que nos hemos reído tantas veces: "ven pa aquí hijo mío que te saco un ojo".

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que en este momento no nombro, esto no hubiese sido posible y que personalmente le he agradecido lo mucho que ha hecho por mí sabiéndolo o sin saberlo, *saps que t'admiro molt i m'alegro de que el context actual hagi reconegut la teva vàlua i esforç*. Gracias a estas dos personas que iniciaron mi camino en este Hospital he podido conseguir realizar esta tesis con dos directores que no pueden ser más adecuados y parecidos a lo que mis orígenes son. Mis dos directores que, aunque no me lo hayan dicho exactamente, con acciones u otro tipo de palabras, veo reflejada la frase que anteriormente he nombrado y que tanto identifica a mi familia y nuestro carácter aragonés de la "franja de ponent". *Moltes gràcies Jordi, per ser com ets i per les teves paraules directes, saps que admiro, encara que sempre "eche la lagrimilla", perquè m'agradaria que algun dia l'orgull t'omplís*. Gracias Joven por tus palabras y tu mirada cuando me ves temerosa que por alguna razón, al mirarte a los ojos veo la calma y seguridad que quieres transmitir. Disculpa mi temor por decir una palabra o afirmación inapropiada que te llevase a avergonzarte de mis razonamientos.

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David, puedes pensar que tú no has sido partícipe de esta vivencia, lo siento, estás muy equivocado, tú has llevado la carga más dura, el periodo final de este esfuerzo, lo siento, no te escaqueas y quiero gritar y que el mundo sepa que sin tu apoyo y tu forma de ser el esfuerzo no sé si hubiese podido dar el fruto que ha dado, eres la persona que necesitaba para ordenar mi mundo y creer en mí misma.

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vivencias con vosotros y de vuestro ser, y cualquier otra cosa sería una falsificación barata de quien soy.

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ABREVIATURAS

ABI: Ankle brachial index

B2M: Beta-2 microglobulina

CCL-2: C-C chemokine ligand 2

CCR-2: C-C chemokine receptor 2

CLI: Critical limb ischaemia

DEPCyMC: 7-*O*-dietil fosforil 3-ciano 4-metil 7-hidroxicumarina

DNPH: 2,4-difenilhidracina

EAC: Enfermedad Arterial Coronaria

EAP: Enfermedad Arterial Periférica

ECV: Enfermedades Cardiovasculares

ELISA: Ensayo por inmunoadsorción ligado a enzimas

HDL: Lipoproteínas de alta densidad

ICAM-1: Molécula de adhesión intercelular 1

LDL: Lipoproteinas de baja densidad

MCP-1: Monocyte chemoattractant protein-1

MO-LDL: LDL mínimamente oxidadas

NO: Óxido nítrico

OX-LDL: LDL oxidadas

O_2^-: Anión superóxido

ONO_2^-: Peroxinitrilo

PCR: Proteína C reactiva

PON: Paraoxonasa

PON1: Paraoxonasa 1

PUFA: Ácidos grasos poliinsaturados

ROS: Especies reactivas de oxígeno

TBBL: 5-tiobutil-butirolactona

TXA2: Tromboxano A 2

VCAM-1: Molécula de adhesión celular vascular 1

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CONTENIDO

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RESUMEN

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Resumen

Las enfermedades cardiovasculares suponen una carga social y asistencial muy importante todavía en la actualidad. La industrialización supuso e instauró cambios en los hábitos alimentarios y estilos de vida, estos hechos conllevaron un cambio en las patologías principales que afectaban a la sociedad y las enfermedades cardiovasculares tomaron la delantera a las patologías predominantes hasta el momento y siguen ocupando el primer lugar actualmente.

La más conocida de ellas y la que está asociada a mayor mortandad sigue siendo la cardiopatía coronaria isquémica, cuya manifestación principal es el infarto agudo de miocardio. A pesar de estos datos, la enfermedad cardiovascular que implica una mayor morbilidad y una carga clínico-asistencial más elevada es la enfermedad arterial periférica.

La mayor limitación de esta patología es su detección puesto que las manifestaciones clínicas asociadas a veces pueden ser confundidas con dolores musculoesqueléticos ya que comparten características, o, incluso, en los estadios iniciales de la enfermedad, estas manifestaciones pueden estar ausentes.

Debido a que la enfermedad arterial periférica conlleva asociada la afectación de un gran número de territorios arteriales, la carga aterosclerótica es significativamente superior a la enfermedad coronaria, y esta peculiaridad ofrece una herramienta muy valiosa para poder evaluar esta enfermedad cuando los síntomas clínicos no son clarificadores o, incluso, cuando son ausentes, en especial desde el punto de vista del análisis de nuevos biomarcadores.

La enfermedad aterosclerótica es una enfermedad inflamatoria, esta inflamación es el producto de la desregulación de los procesos pro-oxidantes y antioxidantes de nuestro organismo, cuyo resultado es el estrés oxidativo.

El estrés oxidativo es uno de los principales causantes de la manifestación aterosclerótica, que incrementa la expresión génica de moléculas inflamatorias, este dato es conocido y actualmente se aplica en el seguimiento de las enfermedades cardiovasculares a través de las guías consensuadas europeas y españolas.

Estos datos nos han llevado a evaluar la posibilidad de evaluar marcadores de estrés oxidativo en la enfermedad arterial periférica, ya que la inflamación es el producto del efecto del estrés oxidativo sobre la expresión de las moléculas inflamatorias.

En nuestro **primer estudio**, publicado en *Free Radical Biology and Medicine* se evaluaron diferentes biomarcadores de estrés oxidativo bajo el supuesto de encontrar aquel que presentase una mejoría en la sensibilidad y especificidad respecto a los biomarcadores inflamatorios utilizados hasta el momento. Los resultados obtenidos han mostrado que los biomarcadores implicados en la peroxidación de lípidos, como son los F2-isoprostanos, y moléculas inflamatorias que presentan un incremento de su expresión por causa del estrés oxidativo, como es el ligando de quimiocinas 2, son los que presentan una sensibilidad y especificidad mayor. Estos resultados son muy alentadores y presentan unos datos de gran valor en el cribaje y control de esta enfermedad; el problema del uso de estos biomarcadores en la medicina asistencial recae en la ausencia de métodos automatizados para la medición de estas moléculas. Este hecho nos llevó a realizar nuestro **segundo estudio**, publicado en *International Journal of Molecular Sciences*, en el que la evaluación se centró en biomarcadores de fácil automatización en los laboratorios clínicos asistenciales. Los datos obtenidos han demostrado que la galectina-3, molécula implicada en procesos inflamatorios en referencia al reclutamiento de células inflamatorias, en combinación con la proteína C reactiva muestran una sensibilidad y especificidad muy elevadas, superior a la combinación de los biomarcadores utilizados en la actualidad, aunque algo inferior a las de las determinaciones de parámetros de estrés oxidativo obtenidas en el primer estudio.

En este segundo estudio también se evaluó la expresión inmunohistoquímica de galectina-3 en las arterias de sujetos sanos y enfermos, mostrando diferencias en la distribución de esta molécula en la estructura arterial, perdiendo expresión en la túnica adventicia e incrementando la expresión en la túnica media y túnica intima en los sujetos que padecen la enfermedad respecto a los sujetos sanos.

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INTRODUCCIÓN

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1. Las enfermedades cardiovasculares:

Los primeros estudios que se refieren al desarrollo de la aterosclerosis los encontramos en 1904 cuando Felix Marchand, utilizando tanto el término arteriosclerosis como aterosclerosis, describió esta patología como la causa más probable de los procesos obstructivos de la mayoría de las arterias (1).

En 1908 encontramos los primeros datos que hacen referencia a la influencia de la alimentación en la aterosclerosis, cuando Alexander Ignatowski, mediante sus estudios experimentales, describió la relación de esta enfermedad con los alimentos ricos en colesterol (2). Fue en 1910 cuando A. Windaus demostró que las lesiones ateroscleróticas contienen 6 veces más colesterol libre y 20 veces más colesterol esterificado que una pared arterial normal (3).

En 1913, Nicolai N. Anichkow, demostró que el colesterol por si mismo causa los cambios en la placa de ateroma establecida en la pared arterial (4). De este autor son conocidos sus estudios sobre los cambios en la fisiopatología cardíaca tales como el estudio descriptivo sobre los ajustes compensatorios en las arterias coronarias cardíacas que se desarrollan en la aterosclerosis (5).

Desde los inicios del siglo pasado las enfermedades cardiovasculares (ECV) fueron incrementando hasta el punto de ser la principal causa de muerte en los países industrializados como consecuencia de los cambios en el estilo de vida tradicionales derivados de la propia industrialización.

No fue hasta finales de la década de los 60 cuando las investigaciones epidemiológicas fueron lo suficientemente concluyentes sobre las causas de las ECV, en ese momento empezaron a surgir las primeras medidas para su prevención y control, las cuales se empezaron a aplicar y, con ello, comenzó a disminuir la prevalencia de las ECV en los países industrializados (6).

Según la definición de la Organización Mundial de la Salud (OMS), las ECV son desórdenes cardíacos y de vasos sanguíneos, los cuales incluyen la cardiopatía coronaria, enfermedad cerebrovascular, arteriopatías

periféricas, cardiopatía reumática, cardiopatía congénita y trombosis venosa profunda y embolias pulmonares (7). Estas patologías son, propiamente, un conjunto de enfermedades que afectan a los diferentes componentes del aparato cardiovascular y comprenden muchos y diferentes procesos fisiopatológicos que se encuentran alterados; la alteración de unos u otros será la que definirá propiamente el tipo de ECV.

Las ECV, en los inicios del siglo XXI, siguen siendo la principal causa de mortalidad en la población adulta. Los datos más recientes de la OMS indican que los países desarrollados, mediante la implantación de programas educacionales de prevención, están consiguiendo una reducción de la mortalidad de hasta el 80%, como es el caso de Finlandia. El porcentaje de muertes por eventos cardiovasculares se sitúa en el 31% a nivel mundial, más del 75% ocurre en países de medianos y bajos ingresos (8), pero se debe tomar en cuenta que estos porcentajes siguen aumentando.

La cardiopatía coronaria isquémica sigue siendo la causa más frecuente de mortalidad debida a las ECV. La etiología inflamatoria de las ECV asociadas a aterogénesis es, hoy por hoy, muy conocida y estudiada (9, 10), motivo por el cual en esta introducción será comentada con más detalle.

2. La aterosclerosis:

El proceso básico y fundamental de la aterosclerosis es el endurecimiento y estrechamiento de las arterias que finaliza con su bloqueo y compromete el flujo sanguíneo, situación que puede causar lesiones nerviosas y tisulares (11, 12).

El inicio y progresión de la aterosclerosis ha sido muy estudiado, la hipótesis inicial que la describía como el simple resultado de la acumulación de lípidos en la pared vascular ha sido desbancada, ya que multitud de datos concluyen que este proceso es mucho más complejo y posee una notable base inflamatoria (13).

En esta figura extraída y adaptada de Arizona Vein and Vascular Center (14, 15) se observa el proceso macroscópico de la aterosclerosis y sus diferentes grados o etapas (Figura 1).

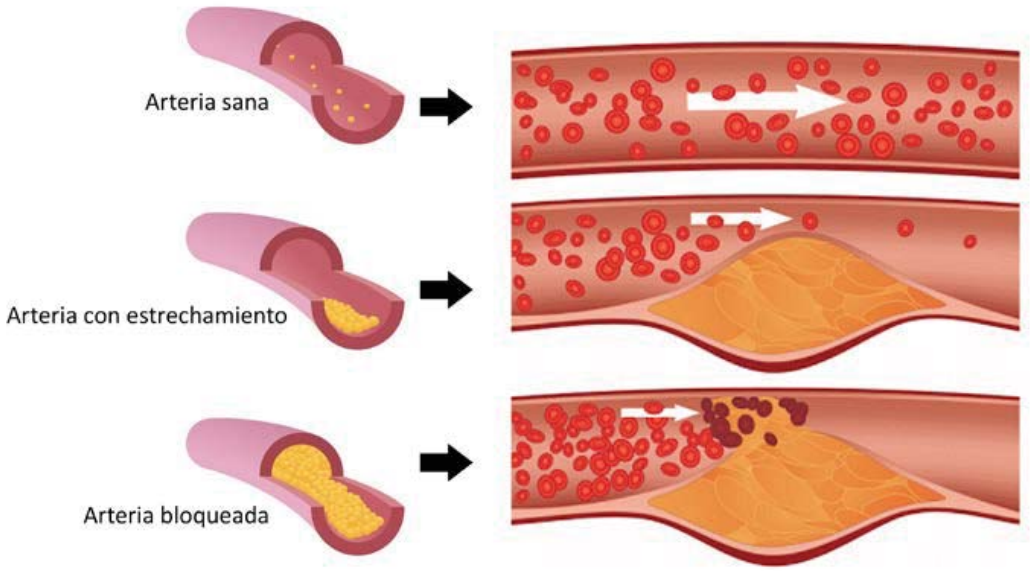

Figura 1: Etapas de formación de la placa de ateroma.

La aterosclerosis es el punto culminante de un proceso que se inicia y transcurre de un modo silente.

Durante el proceso de la aterogénesis se dan una serie de cambios a nivel vascular, llevados a cabo por tres tipos de células inflamatorias, en concreto monocitos, macrófagos y linfocitos T, los cuáles son piezas clave para el desarrollo de esta enfermedad (10, 16, 17). También se debe destacar el papel de las células endoteliales, y la importancia del acumulo de lipoproteínas de baja densidad (LDL) oxidadas en la capa íntima de la arteria, las cuales contribuyen al reclutamiento de los monocitos y su posterior transformación en células espumosas (18, 19).

Estas partículas de LDL oxidadas son retenidas dentro de las células endoteliales, proceso que puede ser una causa o efecto, o ambos, del proceso inflamatorio subyacente. Actualmente se considera que la aterosclerosis comienza con un daño en el endotelio vascular causado por una amplia variedad de factores tales como la presión arterial

elevada, el tabaquismo, una concentración elevada de colesterol, la diabetes y la hiper-homocisteinemia, entre otros (13, 20, 21).

La activación de las células endoteliales es el factor que permite la atracción, adhesión y posterior migración a través del endotelio de las células inflamatorias; a su vez, la propia disfunción endotelial juega un papel fundamental en el desarrollo de la aterosclerosis localizada.

La formación de la placa se inicia por un daño o disfunción endotelial en el lumen de la pared arterial. A continuación, las LDL pasan a través del endotelio dañado y se depositan dentro de la pared arterial. El fenómeno clave en el desarrollo de la aterosclerosis es la oxidación de las LDL, proceso que genera especies reactivas de oxígeno (ROS) y su producto final es la transformación de los macrófagos en células espumosas (22) (Figura 2).

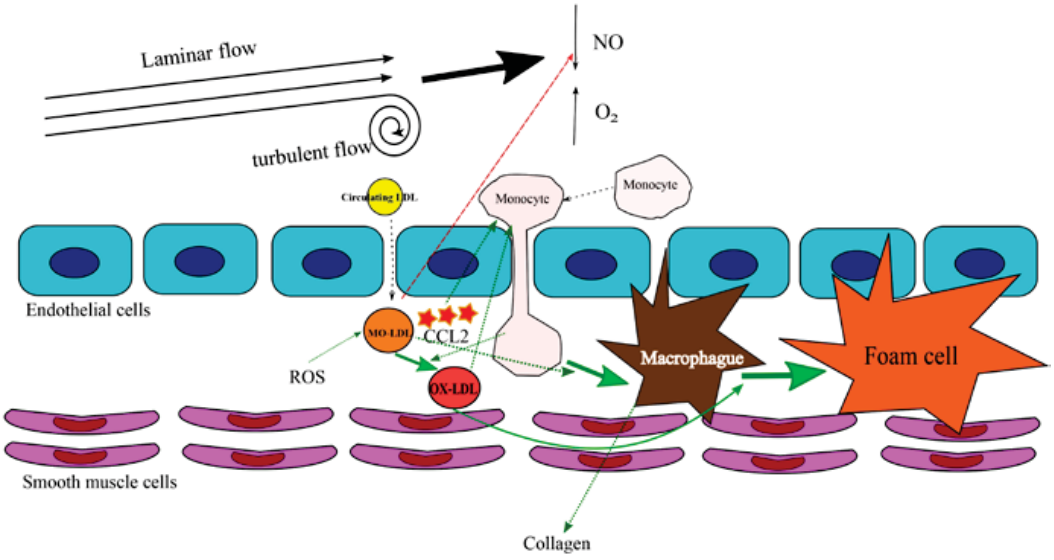

Figura 2: Entrada de circulación de LDL, transformación en MO-LDL, transformación en OX-LDL y formación de placa aterosclerótica.

Las ROS son especies químicas que contienen un electrón desapareado en el orbital más externo de la molécula de oxígeno, situación que les confiere una elevada reactividad biológica y que se produce en el metabolismo aeróbico normal en circunstancias tales como el procesamiento de los alimentos, la respiración o el ejercicio físico.

La producción de ROS está controlada estrictamente por el organismo gracias a la ayuda de antioxidantes endógenos y exógenos. Si las ROS no son controladas pueden oxidar y dañar moléculas esenciales tales como los ácidos grasos poliinsaturados (PUFA), DNA, proteínas y carbohidratos, dirigiendo el proceso, de este modo, hacia un daño en la estructura celular y el funcionamiento de la propia célula que finalmente desemboca en la muerte de la misma (23, 24).

El desequilibrio entre la producción de ROS y los mecanismos de control antioxidante se denomina estrés oxidativo.

La medición directa de ROS es difícil debido a que estas moléculas, en general, tienen una vida media muy corta; es por esta razón que, habitualmente, el estrés oxidativo se evalúa indirectamente mediante la medición de una determinada función o actividad específica de biomarcadores medibles.

Los PUFA están presentes en las partículas de LDL y son particularmente sensibles a la oxidación. La peroxidación lipídica de los PUFA tiene unas características generales idénticas a las descritas para los procesos oxidativos en los lípidos de las membranas celulares. La peroxidación de los ácidos grasos, a nivel molecular, es una reacción en cadena que consiste en tres etapas:

1. En una primera etapa se produce un radical de ácido graso cuando alguno de éstos reacciona con las ROS.

2. En una segunda etapa, conocida como propagación, los radicales de ácidos grasos, de los cuales es conocida su elevada inestabilidad, reaccionan con oxígeno molecular dando lugar a un radical peróxido de ácido graso. Estos radicales peróxido de ácido graso son especies altamente reactivas que pueden interaccionar con otros ácidos grasos iniciando una reacción en cadena.

3. La tercera etapa sólo ocurre cuando las concentraciones de ROS son suficientemente elevadas y consiste en la reacción entre dos radicales para producir especies estables (25). Este proceso juega un papel clave en el inicio y progresión de la aterosclerosis (26).

Estos datos explican el proceso aterosclerótico como el producto de la desregulación entre los procesos pro-oxidantes y antioxidantes, cuyo resultado es el estrés oxidativo.

Cuando las partículas de colesterol LDL atraviesan el endotelio dañado son parcialmente oxidadas por las ROS, originando las llamadas LDL mínimamente oxidadas (MO-LDL). Las MO-LDL estimulan las células endoteliales y las células del músculo liso, las cuáles promueven la síntesis de la molécula ligando de quimiocinas 2 (CCL2), cuyo nombre anglosajón es *chemokine (C-C motif) ligand 2*, anteriormente denominada proteína quimioatrayente o quimiotáctica de monocitos 1 (MCP1) (27), que dirige la migración de leucocitos específicos del torrente sanguíneo hacia los tejidos lesionados.

Las ROS también estimulan la expresión de las moléculas de adhesión celulares tales como la molécula de adhesión intercelular 1 (ICAM-1 o CD54) y la molécula de adhesión celular vascular 1 (VCAM-1), que a su vez también promueven la producción de moléculas de adhesión de leucocitos, las cuales incrementan la adhesión de monocitos y linfocitos a las células endoteliales.

Estos datos nos permiten enfatizar que el papel de los linfocitos, monocitos y macrófagos en la patogenia de la aterosclerosis es consecuencia de la respuesta a los diferentes estímulos o factores que conducen hacia el proceso aterosclerótico derivados de la disfunción vascular causada por las anomalías a nivel lipídico, consecuencia de la desregulación en los procesos antioxidantes y pro-oxidantes (22).

Cuando los monocitos se adhieren a la superficie de las células endoteliales, las MO-LDL son completamente oxidadas a OX-LDL. La oxidación completa de las LDL desde MO-LDL es catalizada, principalmente, por la mieloperoxidasa (un enzima que se expresa en los macrófagos encontrados en las lesiones ateroscleróticas) y las glicosilasas (28-30). Algunos estudios han sugerido que la oxidación enzimática de las LDL es posible *in vivo* gracias a la interacción con la pared arterial, las células sanguíneas, los constituyentes plasmáticos y los componentes de la matriz de la pared arterial, que son capaces de hidrolizar los esteres de colesterol, fosfoglicéricos y triglicéridos.

Los PUFA son oxidados por las lipo-oxigenasas de los macrófagos, proceso que desencadena la formación de hidro-peróxidos de ácidos grasos de LDL. De un modo similar, las oxidasas de colesterol son capaces de generar LDL que se enriquece con productos del colesterol oxidado (26, 31).

Las MO-LDL inducen una respuesta inflamatoria que conduce a la apoptosis mediante la activación de factores nucleares, estimulación de la coagulación, inducción de la peroxidación en lesiones e inhibición de la producción de óxido nítrico (NO). De un modo similar, las OX-LDL producen una reacción inflamatoria que conduce a la infiltración de linfocitos T. Ambas moléculas oxidadas, MO-LDL y OX-LDL, son inmunogénicas y son capaces de estimular la liberación de autoanticuerpos mientras que, a su vez, pueden alterar la agregación plaquetar. Tanto las OX-LDL como CCL2 promueven la migración de monocitos hacia el subendotelio. Además, las OX-LDL son capaces de inducir la producción de moléculas pro-inflamatorias tales como la Selectina P, VCAM-1, ICAM-1 y CCL2 (32).

El proceso inflamatorio depende, principalmente, de dos moléculas de adhesión: las selectinas (las cuales están implicadas en la deposición inicial de los leucocitos en el endotelio) y las inmunoglobulinas (que son responsables de la adhesión de los leucocitos). Hoy en día, sin embargo, no está clara la evidencia sobre los factores responsables del incremento de la expresión local de moléculas de adhesión y citocinas; a pesar de ello, sabemos que cuantas más moléculas LDL se oxidan más moléculas pro-inflamatorias se generan (32).

La diferenciación de monocitos a macrófagos es debida a la liberación del factor estimulante de colonias de monocitos de las células endoteliales por las MO-LDL. Los macrófagos diferenciados desarrollan un receptor para las OX-LDL, por lo que estas moléculas son capturadas por los macrófagos vía fagocitosis, para, de este modo, formar las células espumosas. Todo este proceso es el que desemboca, finalmente, en la necrosis tisular (26).

Las OX-LDL exacerban el daño intracelular endotelial, mientras que, al mismo tiempo, la ausencia de NO genera vasoconstricción, puesto que

las funciones de esta molécula son la regulación el tono vascular e inhibición de la agregación plaquetar (33). El NO deriva de la L-arginina y se genera y libera por las células endoteliales, tiene funciones a nivel transcripcional mediante la modulación de la ruta de señalización del factor nulcear Kappa B (NF-κB) y mediante la inhibición de la expresión de los genes *VCAM-1* en las células endoteliales (34).

Los factores más importantes implicados en la progresión de la aterosclerosis son, por una parte, la proliferación de las células del músculo liso, por otra parte la migración de monocitos para su transformación en macrófagos junto con la síntesis del tejido conectivo y matriz extracelular (31). Los macrófagos generan muchos factores de crecimiento involucrados en la formación de colágeno, fibras elásticas y proteínas.

Otro mecanismo causante de la oxidación de las partículas LDL es la proteína rica en cobre, ceruloplasmina. Esta proteína en presencia del anión superóxido (O_2^-) promueve la oxidación de la LDL, causa y efecto de la aterosclerosis. Aunque estos datos parecen apuntar a que la ceruloplasmina juega un papel importante en la oxidación de las LDL no está claro su mecanismo de acción (35).

A pesar de todos estos mecanismos disruptivos, el organismo tiene diversos sistemas para contrarrestar las alteraciones mediadas por el estrés oxidativo. Bajo condiciones de flujo sanguíneo laminar la sintasa de NO está incrementada junto con los niveles de NO; con ello, sus acciones antiinflamatorias y vasodilatadoras también lo están. Los niveles incrementados de NO son el primer mecanismo de defensa que tiene nuestro organismo frente a la formación de la placa de ateroma cuyas dos funciones antiaterogénicas fundamentales son la relajación de los vasos e inhibición de la agregación plaquetar (31, 36).

Bajo condiciones de flujo turbulento, tales como las que ocurren en las ramas arteriales o cuando hay una disfunción en el endotelio, existe una inhibición de la síntesis de NO, un incremento de la expresión de VCAM-1 y la transformación de los monocitos a células espumosas (34, 37). No sólo el flujo turbulento induce la contracción vascular, las ROS tienen un efecto similar; el oxígeno (O_2) y el peróxido de hidrógeno (H_2O_2) tienen la

capacidad de producir contracciones en los vasos mediante dos mecanismos posibles, uno directo (11) y uno indirecto mediado por la endotelina-1 o por la descomposición del NO (38-40).

Pero, a su vez, las ROS pueden inducir un flujo turbulento directamente mediante el daño de las células endoteliales y reduciendo la producción del NO, la disminución de la producción de NO se suma a la disminución de su concentración debido a que el O_2 puede reaccionar con NO para producir más ROS activas tales como el peroxinitrilo (ONO_2^-) o O_2^-. Las ROS producidas, al mismo tiempo, pueden reducir la síntesis de prostaciclina y disminuir la generación de vasodilatadores en el endotelio. El resultado es un incremento en la circulación de catecolaminas, que causan la vasoconstricción y un incremento de la presión sanguínea (26, 31).

Otro mecanismo que causa vasoconstricción es el mediado por la glucosa que incrementa la actividad del sistema simpático y actúa elevando la presión sanguínea.

Todos estos datos demuestran que las ROS, mediante diferentes mecanismos, pueden inducir hipertensión y aterosclerosis.

El segundo mecanismo de defensa frente a la producción de la placa de ateroma viene proporcionado por las lipoproteínas de alta densidad (HDL), las cuales penetran dentro del subendotelio y actúan como el instrumento de unión y eliminación de las moléculas de colesterol intracelulares. En la aterogénesis este mecanismo no es funcional debido a que las HDL son insuficientes para eliminar el exceso de LDL que penetran en la célula. Hoy en día existen diversos estudios que han demostrado que las partículas HDL protegen las partículas LDL de la oxidación mediante dos apolipoproteinas con actividad enzimática: la paraoxonasa 1 (PON1) y la acetilhidrolasa del factor activador de plaquetas (27, 41, 42).

La PON1 es una hidrolasa capaz de degradar un amplio espectro de sustratos, que circula por el plasma unida a las HDL y es capaz de hidrolizar los fosfolípidos presentes en las LDL oxidadas (26).

El tercer mecanismo de defensa está proporcionado por las plaquetas que se adhieren al endotelio dañado y liberan factores de crecimiento conduciendo a la formación de tejido conectivo.

Las células espumosas representan el estadio más inicial reconocible de la aterogénesis, el cual es reversible. Los detritos de colesterol y la acumulación de células espumosas en el subendotelio es el origen de la placa aterosclerótica. En la placa aterosclerótica más avanzada tiene lugar la acumulación de células espumosas; la capa íntima del endotelio se vuelve progresivamente más fibrosa mientras que las células del músculo liso se acumulan dentro de la lesión y se producen macromoléculas extracelulares que forman la matriz fibrosa. El calcio también se acumula en la placa aterosclerótica, la cual progresa con la producción de proteínas por las células del músculo liso vascular.

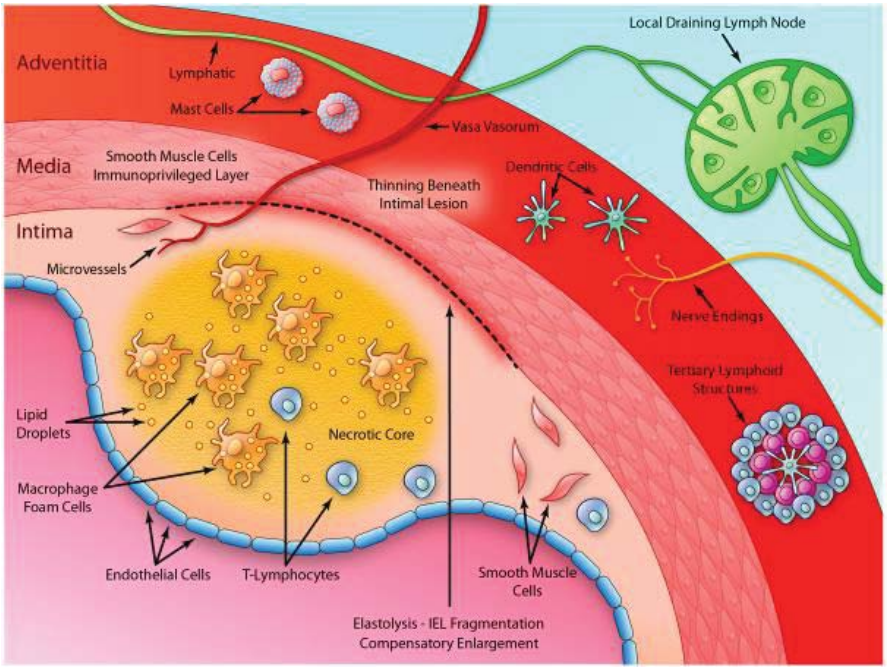

Figura 3: Lesión aterosclerótica, respuesta inmune e inflamatoria en las diferentes capas del vaso. Figura adaptada de Libby et al. (43).

3. La enfermedad arterial periférica: características clínicas y epidemiológicas:

El término Enfermedad Arterial Periférica (EAP) se usa con frecuencia en la literatura médica pero existen variaciones considerables de la definición respecto a las categorías de enfermedades incluidas, por ejemplo la aterosclerosis, la displasia fibromuscular y la vasculitis; también respecto a los territorios arteriales afectados.

La EAP, a su vez, es conocida con diferentes nombres: valvulopatía periférica, aterosclerosis obliterante, claudicación intermitente, enfermedad vaso-oclusiva periférica e insuficiencia arterial periférica, entre otros (44, 45).

En este trabajo se enfocará la EAP desde la definición clínica que compete el acumulo de colesterol en las arterias de las extremidades inferiores, propiamente. En la práctica clínica, en determinadas ocasiones, se incluye también la enfermedad venosa y linfática (44).

La EAP que afecta a las extremidades inferiores es un problema de salud muy importante que puede desembocar en eventos catastróficos en los diferentes territorios arteriales tales como las carótidas extracraneales, las arterias de las extremidades superiores, las arterias mesentéricas y renales y, por supuesto, las arterias que pertenecen a las extremidades inferiores (46).

Esta enfermedad tiene un amplio espectro de manifestaciones clínicas, desde aquellos individuos que son asintomáticos hasta aquellos que tienen síntomas clínicos en los miembros inferiores tales como claudicación intermitente o incluso la pérdida de tejido. La obstrucción total o parcial de las arterias de las extremidades inferiores está causada por una mala o deficiente circulación, lo que deriva en la conocida claudicación intermitente caracterizada por dolor en los músculos de las piernas durante la deambulación, este dolor desaparece unos minutos después de haber cesado la actividad. Este síntoma no debe confundirse con el dolor causado por un nervio espinal pinzado o dañado, en cuyo caso el dolor no desaparece cuando cesa la actividad (46).

La progresión de la enfermedad causa dolor, incluso en reposo, y éste se acentúa cuando se elevan las piernas. El dolor puede aparecer en las pantorrillas o también en los pies.

Los estadios más graves de la EAP se asocian con hipoperfusión, que se traduce en dificultad en la cicatrización de las heridas; este proceso puede progresar a isquemia, ulceración o incluso gangrena. Un tercio de los pacientes en esta situación finalizarán con la amputación del miembro (46). La presencia de dolor en reposo, úlceras y gangrena que finaliza en la pérdida de tejido se conoce como isquemia crítica de las extremidades (CLI "critical limb ischaemia"), ésta es la manifestación clínica más grave de la EAP, según los datos de un estudio llevado a cabo en Suecia en la primera década del siglo XXI, se estima que la prevalencia de esta manifestación es del 0.4% en personas que superan los 60 años (47).

La incidencia anual mundial de CLI se estima entre 500 y 100 nuevos casos por cada millón de habitantes; debemos remarcar que la incidencia en pacientes con *diabetes mellitus* es superior a la estimada en la población general (48).

La posibilidad de sufrir EAP incrementa con la edad; de este modo encontramos que cuando establecemos el punto de corte de edad en 55 años la prevalencia de EAP de extremidades inferiores es, aproximadamente del 20%. La manifestación clínica de la EAP dependerá de la cantidad de territorios arteriales afectados o carga de aterosclerosis.

En aquellos pacientes que tienen manifestaciones clínicas, generalmente la carga aterosclerótica de la enfermedad suele ser muy superior que en aquellos que no tienen eventos. A pesar de ello, aquellos sujetos que tienen una vida sedentaria y afectaciones en una única zona suelen ser oligosintomáticos o incluso, asintomáticos, pero debemos tener presente que las medidas preventivas sólo son eficaces en estadios iniciales de la enfermedad y es por esta razón resulta tan importante la detección cuando se inicia esta patología (9, 49, 50).

Los estadios iniciales de la EAP son habitualmente asintomáticos, tal y cómo se ha remarcado, esta condición es el motivo por el cual las medidas preventivas se aplican demasiado tarde. Las guías españolas de la EAP de 2012 estimaban que 16% de la población americana y europea presenta EAP, lo que supone más de 27 millones de personas afectadas. Pero los datos más alarmantes son que de todas ellas, más de 17 millones presentan la enfermedad de manera asintomática.

Las guías europeas de la EAP (48) estiman que en un rango de edad de población entre 60 y 90 años, un 18% sufre esta enfermedad, pero tan solo un 7% son sintomáticos; por suerte, únicamente un 0.4% sufren CLI. Los datos estadounidenses brindados por la CDC en la revisión de 2015 (51), estiman que aproximadamente 8.5 millones de personas en Estados Unidos sufren EAP, entre 12 y 20% de los sujetos que la padecen tienen más de 60 años, pero se estima que tan solo entre un 70 y 80% de los pacientes les ha sido pautada una terapia antiplaquetar o hipolipemiante. La EAP está asociada a una elevada morbilidad y mortalidad y, lo que es más preocupante, su prevalencia incrementa dramáticamente con la edad.

En un futuro nada lejano se espera que la prevalencia de esta enfermedad incremente en Europa y Asia (52) hasta los niveles en los que se encuentra Estados Unidos.

Los datos más recientes del meta-análisis que engloba 34 estudios, publicado por Fowkes et al. (44), estiman que existen 202 millones de personas afectadas por EAP en todo el mundo. Este análisis indica un incremento de la prevalencia de 23.5% en la primera década del siglo XXI (53).

La evaluación clínica de la EAP necesita tener en cuenta diferentes aspectos de la enfermedad: la historia, epidemiología y los factores de riesgo (54), que son factores predictivos de la progresión de la enfermedad si no son redirigidos.

Los factores de riesgo de la aterosclerosis en la EAP son similares a aquellos que se presentan en la enfermedad arterial coronaria (EAC), los más importantes son: la raza (pertenecer a la raza negra se asocia a

duplicar el riesgo de padecer EAP comparado con la raza blanca), la edad (la población de más edad tiene mayor riesgo de sufrir esta enfermedad comparado con miembros más jóvenes) y el sexo (pertenecer al sexo masculino conlleva un mayor riesgo de sufrir EAP que pertenecer al sexo femenino)(44).

Otros factores de riesgo prematuro para sufrir EAP son el hábito tabáquico, la diabetes, la hipertensión, un colesterol circulante elevado, triglicéridos elevados y la hiperhomocisteinemia. Debemos tener en cuenta que las concentraciones elevadas de fibrinógeno se consideran un factor de riesgo de padecer un evento trombótico, y un hematocrito elevado promueve la hiper-coagulación (55), factores relacionados también con la EAP.

Actualmente el cociente colesterol HDL respecto al colesterol total es el mejor índice de predicción bioquímico para la EAP, de acuerdo con el documento consenso (TASMCII) para el control de la EAP (54).

Los estudios epidemiológicos indican que la prevalencia de la EAP en la población mundial general se sitúa entre el 3 y el 10%, pero se eleva al 20% si se estima la prevalencia de esta enfermedad en personas de más de 55 años.

Estos resultados indican que las diferentes comorbilidades se asocian a la edad, básicamente por un incremento del estrés oxidativo (46, 54, 56).

El índice tobillo-trazo (ABI por sus siglas en inglés: ankle brachial index) ha sido muy utilizado y aún lo es hoy en día como método de cribado para establecer y evaluar la gravedad de la EAP.

Índice ABI (48, 57)(Tabla 1):

Valor ABI	**Interpretación**
>1.3	Anormal, vaso no compresible (calcificado)
1.0-1.29	Normal
0.91-0.99	Indeterminado
0.89-0.91	EAP leve
0.41-0.89	EAP moderada
<0.4	EAP grave

El ABI compara la presión sistólica en las extremidades superiores respecto a aquella existente en las extremidades inferiores. Mediante un ultrasonido Doppler y un esfingomanómetro, se mide la presión sanguínea en el brazo y en la pierna correspondiente. El índice obtenido refleja el porcentaje o extensión de la EAP (58).

El ABI es una clasificación efectiva y objetiva, los valores reducidos de ABI confirman el diagnóstico de EAP y detectan EAP significativas en pacientes sedentarios y asintomáticos. Este índice se usa en el diagnóstico diferencial en aquellos pacientes que presentan síntomas en extremidades inferiores para determinar la etiología vascular de los mismos, en la identificación de pacientes con EAP con disminución de la funcionalidad en las extremidades inferiores (incapacidad para caminar una distancia definida o a una velocidad estándar) y, finalmente, también proporciona información clave sobre el pronóstico a largo plazo de la propia enfermedad. Un ABI de 0.9 se asocia con un riesgo de 3 a 6 veces superior de mortalidad por enfermedad cardiovascular (31, 59). Sin embargo, el ABI tiene un gran inconveniente cuando se aplica en pacientes diabéticos o pacientes con fallo renal, ya que éstos tienen arterias calcificadas en las extremidades inferiores y, por ello, el ABI está falsamente elevado. En estos casos la presión arterial se debe medir en los dedos de los pies en relación con la medida en el brazo.

Existen otras clasificaciones para estratificar el grado de EAP, la más ampliamente utilizada es la clasificación de Fontaine; pero existen otras clasificaciones, por ejemplo la clasificación Rutherford.

Índice Fontaine (48)(Tabla 2):

Clasificación de Fontaine	
Estadío	**Manifestación clínica**
I	Asintomático
II a	Claudicación leve (en distancias > 200 metros)
II b	Claudicación moderada-grave (en distancias < 200 metros)
III	Dolor isquémico en reposo
IV	Ulceración o gangrena

Índice Rutherford (48)(Tabla 3):

Clasificación de Rutherford		
Grado	**Estadío**	**Manifestación clínica**
0	0	Asintomático
I	1	Claudicación leve
I	2	Claudicación moderada
I	3	Claudicación grave
II	4	Dolor isquémico en reposo
III	5	Pérdida menor de tejido
III	6	Pérdida mayor de tejido

La arteriografía es todavía, hoy en día, la técnica de referencia para el diagnóstico de la EAP, por esta razón todas las clasificaciones utilizadas actualmente deben ser comparadas respecto a ésta; sin embargo, la arteriografía es una técnica semi-invasiva que sólo debe aplicarse en aquellos pacientes que sean candidatos a una cirugía o intervenciones percutáneas, una de sus limitaciones estriba en la infraestimación respecto a la extensión de la aterosclerosis en vasos de apariencia normal (60), por lo que la utilidad en la clasificación de esta enfermedad se ve limitada.

Se debe tener muy en cuenta que arteriografía no debe ser aplicada en pacientes que padezcan enfermedad renal puesto que el contraste utilizado en esta técnica puede causar efectos adversos en este grupo de pacientes y, por tanto, está contraindicado, especialmente en aquellos que sufran una enfermedad renal en estado avanzado (61, 62).

4. Biomarcadores en la enfermedad arterial periférica:

Un biomarcador es una molécula que encontramos en circulación en concentraciones fisiológicas, la cual tiene unas características biológicas que le confieren la propiedad de ser medida y evaluada; el objetivo de un biomarcador es proporcionar la capacidad para evaluar los diferentes

aspectos de una enfermedad más allá de los síntomas clínicos. Cuando un biomaracador indica susceptibilidad a padecer una enfermedad se denomina factor de riesgo, cuando indica capacidad para discriminar los sujetos afectados por la enfermedad de aquellos que no lo están se denomina biomarcador de cribado, cuando discrimina la progresión de una enfermedad se denomina biomarcador de estadiaje, cuando estima la progresión de la enfermedad se denomina biomarcador pronóstico y, finalmente, cuando permite evaluar la eficacia de un tratamiento, se denomina biomarcador de tratamiento.

La precisión de un biomarcador se suele evaluar mediante las curvas ROC (Receiver Operating Characteristics) las cuales representan sensibilidad frente a la especificidad de un determinado biomarcador.

En lo referente a la EAP encontramos dos grupos de biomarcadores, los biomarcadores clásicos y los biomarcadores emergentes.

Dentro del grupo de biomarcadores clásicos encontramos la beta-2 microglobulina (B2M) y la proteína C reactiva medida mediante métodos de alta sensibilidad (PCR).

El grupo de biomarcadores emergentes como tal, es un grupo condicionado por las nuevas aplicaciones e investigaciones realizadas en el terreno del desarrollo tecnológico en la medición de moléculas conocidas pero de menor aplicación en el diagnóstico de la EAP, y por ello es un grupo dinámico.

4.1. Biomarcadores clásicos

Los biomarcadores clásicos son moléculas comúnmente utilizadas en el laboratorio clínico para el estudio de la inflamación.

Se utilizan en la práctica clínica diaria para el cribaje, estadiaje, pronóstico y estudio de la progresión de diversas enfermedades inflamatorias además de la EAP.

4.1.1 Proteína C reactiva:

La proteína C reactiva (PCR) es un reactante de fase aguda sintetizado en el hígado. Las concentraciones séricas de este biomarcador aumentan en estados de inflamación o infección y también pueden asociarse a eventos

tales como infarto de miocardio, intervención quirúrgica o trauma (63, 64).

El método de alta sensibilidad para la medición de la PCR permite determinar concentraciones realmente bajas de esta proteína, y es muy útil para establecer la presencia de inflamación y/o riesgo de enfermedad cardiovascular (CVD). Este biomarcador se suele utilizar en combinación con otros marcadores tales como el colesterol, triglicéridos, lipoproteína-a (Lp_a) para la determinación del riesgo de ECV o EAP.

La American Heart Asociation (AHA) y el Center for Disease Control and Prevention (CDC) definieron los siguientes grupos de riesgo de ECV en función de las concentraciones de PCR (65):

Bajo riesgo: PCR < 1.0 mg/L

Riesgo intermedio: PCR 1.0-3.0 mg/L

Riesgo elevado: PCR >3.0 mg/L

4.1.2. β-2-microglobulina:

La beta-2-microglobulina (B2M) es una proteína de bajo peso molecular que se encuentra en la superficie de las células nucleadas, pertenece al complejo mayor de histocompatibilidad de clase I y se libera a la circulación por las células nucleadas, especialmente por los linfocitos B y células tumorales.

En condiciones normales se excreta en muy bajas concentraciones en orina, un 99% de la B2M filtrada se reabsorbe por los túbulos renales. Las concentraciones elevadas de B2M en orina son indicativas de una disminución en el aclaramiento de la misma, y, con ello de enfermedad tubular renal; mientras que, ante un incremento de la concentración de B2M sérica, debe hacer sospechar en un incremento en su producción, cuyas causas más probables son una infección sistémica, enfermedad autoinmune o enfermedad hematológica maligna.

Los enfermos con EAP presentan elevadas concentraciones séricas de B2M debido a un incremento de su producción a causa de la acrecentada rigidez arterial e inflamación de los vasos debido a los repetitivos episodios de isquemia y reperfusión en las piernas, y por una disminución

del aclaramiento renal debido a la disfuncionalidad de las arterias renales (61, 66, 67).

La rigidez arterial reduce la capacidad de tamponamiento de las arterias elásticas, esta situación incrementa la presión y pulso sistólicos y promueve la hipertrofia ventricular izquierda y su disfunción, alterando, de ese modo, la capacidad miocárdica de perfusión. Esta anomalía juega un papel muy importante en la aterogénesis de la EAP (68).

4.2. Biomarcadores emergentes

Los biomarcadores emergentes son moléculas menos investigadas y cuyas aplicaciones en el laboratorio clínico se están proponiendo recientemente.

La relación del estrés oxidativo con la aterosclerosis ha sido descrita y demostrada en apartados anteriores. La desregulación entre los procesos oxidantes y los mecanismos antioxidantes da lugar al estrés oxidativo, su relación con la aterosclerosis se ha descrito a diferentes niveles y, es por esta razón por la cual la investigación en torno a biomoléculas o biomarcadores que nos permitan efectuar una medida de dicho estrés tiene una gran relevancia en este estudio.

4.2.1 F-2 Isoprostanos

Los isoprostanos son una familia de eicosanoides de origen no enzimático, derivados del ácido araquidónico y producidos por oxidación aleatoria. Estas moléculas son generadas mediante oxidación *in situ* por las ROS a partir de los grupos acilo que contienen los fosfolípidos de membrana. Los isoprostanos tienen efectos muy importantes a nivel vascular (69).

El F2-isoprostano (figura 3), que dispone de diferentes regioisómeros de las series 5, 12, 8 ó 15 según cuál sea el átomo de carbono al que se encuentre unido el grupo hidroxilo de la cadena lateral, y que puede denominarse también como 15-F2t-isoprostano, 8-iso-15(S)-Prostaglandin $F_{2\alpha}$, 8-iso-15(S)-Prostaglandin $F_{2\alpha}$, 8-*iso*$PGF_{2\alpha}$, $iPF_{2\alpha}$-III o 8-*epi* $PGF_{2\alpha}$, es un vasoconstrictor renal y pulmonar muy potente y se ha descrito su asociación como mediador causal del síndrome hepato-renal y la toxicidad pulmonar por oxígeno (70, 71).

Los isoprostanos han sido propuestos como un marcador de deficiencia antioxidante y estrés oxidativo (72, 73).

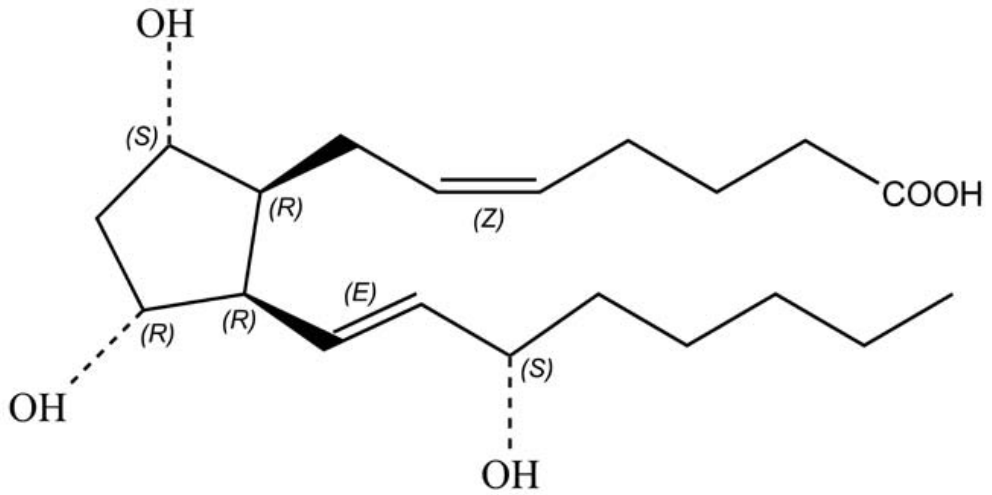

Figura 3: F2-isoprostano (chemdraw.cdx Perkin Elmer software).

Estas moléculas tienen diferentes modos de acción, pueden inhibir la angiogénesis mediante el bloqueo de la migración de las células endoteliales, inducida por el factor de crecimiento endotelial vascular (VEGF), y el bloqueo de la formación de vasos cardíacos. Estas situaciones estimulan la síntesis de factor de crecimiento tumoral β (TGF-β), el cual se encuentra implicado en la nefropatía asociada a la diabetes de tipo I.

La actividad de los isoprostanos se bloquea por los antagonistas del receptor de tromboxano A_2 (TXA2), indicando, por tanto, que los isoprostanos estimulan la biosíntesis de TXA2.

En el plasma de los individuos sanos encontramos un equilibrio entre los mecanismos pro-oxidantes y antioxidantes, y con ello, es completamente normal encontrar concentraciones bajas de F2-isoprostanos, entre 3 y 22 pg/mL.

Los isoprostanos son productos estables de la peroxidación lipídica (74) y se les considera los analitos más sensibles y reproducibles en lo que se refiere a la cuantificación de la peroxidación y el estatus de estrés oxidativo *in vivo* (75).

Estas moléculas tienen una distribución ubicua en plasma, orina, lavado broncoalveolar, líquido cefalorraquídeo, bilis y otros tejidos. La matrices más apropiadas para su cuantificación son el plasma y la orina debido a que pueden ser obtenidas sin procedimientos invasivos y sus valores son representativos de la producción endógena y, con ello, proporcionan un valor preciso de la carga de estrés oxidativo *in vivo* (76).

La determinación de los isoprostanos se ha estudiado en diversas enfermedades, incluidas aterosclerosis, diabetes, obesidad y enfermedad pulmonar. Existen diferentes métodos para su cuantificación en los fluidos biológicos incluida la cromatografía de gases/espectrometría de masas de ionización química ión-negativo (GC/MS), cromatografía líquida/espectrometría de masas (LC/MS), inmunoensayos e inmunosensores electroquímicos (76).

Los métodos de cuantificación de F2-isoprostanos mediante cromatografía de gases son los recomendados debido a su elevada especificidad, pero entre sus desventajas encontramos el elevado coste y la laboriosa preparación de la muestra; la cromatografía líquida ofrece ventajas similares, la preparación de la muestra no resulta tan complicada, pero aún con eso, este método resulta caro y laborioso. Por esta razón los métodos alternativos, tal y cómo lo es el ELISA (ensayo por inmunoadsorción ligado a enzimas) siguen siendo usados y ofrecen resultados de calidad con una buena correlación respecto a los métodos cromatográficos (77), estos métodos son más baratos y simples, aunque su desventaja estriba en su mayor imprecisión e inexactitud. Se debe destacar que el desarrollo de los inmunosensores electroquímicos para la determinación de los isoprostanos supone una serie de ventajas respecto al ELISA tales como una mejor precisión, unos límites de detección menores y un tiempo de ensayo menor, pero la utilización de estos electrodos no está muy extendida, a pesar de sus ventajas (78).

4.2.2 Paraoxonasa 1

La paraoxonasa 1 (PON1) es una proteína que pertenece a una familia de enzimas compuesto por PON1, PON2 y PON3 que derivan de un precursor común cuyos genes se encuentran en el brazo q del cromosoma 7 (21.3-22.1) (79).

En la especie humana PON1 y PON3 se expresan en la mayoría de tejidos, especialmente en hígado, riñón y epitelio y se encuentran en plasma unidas a las HDL. PON2, en cambio, se expresa exclusivamente en tejidos y es un enzima intracelular que no puede ser medido en circulación (20).

PON1 tiene actividad de defensa frente xenobióticos gracias a que posee actividad lactonasa, paraoxonasa y arilesterasa; en cambio PON2 y PON3 únicamente tienen actividad lactonasa y prácticamente nula actividad paraoxonasa y arilesterasa (80).

Los tres enzimas son capaces de degradar los peróxidos lipídicos, fruto de la oxidación de fosfolípidos, en las moléculas de LDL y HDL y moléculas de colesterol de las membranas celulares. Esta función pone de manifiesto las propiedades antiinflamatorias y ateroprotectoras de estos enzimas (27, 81).

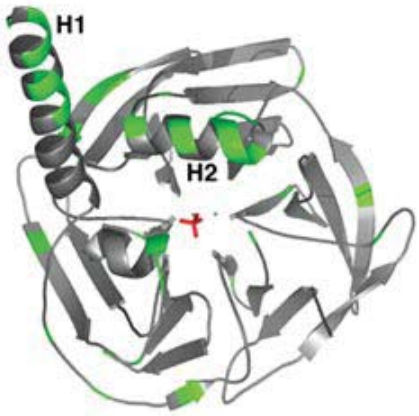

Figura 4: Estructura terciaria de PON1. Figura adaptada de Harel M. et al (82).

Un método ampliamente utilizado para la determinación de la actividad PON1 es la medida de la capacidad de hidrolítica del paraoxón a 37 ºC en un buffer 0.05 mM de glicina a pH 10.5 con 1mM de $CaCl_2$, la lectura se realiza a una longitud de onda de 410nm (83). El paraoxón es una sustancia extremadamente tóxica e inestable que requiere una rápida preparación bajo condiciones de seguridad importantes.

Otro sustrato que puede utilizarse en la medición de la actividad de PON1 es el fenilacetato, menos tóxico que el paraoxón pero que también requiere precaución en su manipulación. La medición de su actividad arilesterasa se realiza en las mismas condiciones pero a una longitud de onda de 270 nm, rango al que no todos los espectofotómetros pueden llegar (20).

Asimismo, la capacidad hidrolítica de las lactonas o actividad lactonasa de PON1 se puede aprovechar medir la actividad de este enzima, y para ello se utiliza como sustrato la 5-tiobutil-butirolactona (TBBL). La medición de la actividad lactonasa se realiza a 25 ºC y en una longitud de onda de 412 nm mediante la preparación de una solución que contiene CaCl2 1 mM, TBBL 0.25mM y 0.5 mM 5,5'-dithio-bis-2-ácido nitrobenzóico (DTNB), en 0.05 mM tampón Tris-HCl a pH=8.0. Esta actividad se correlaciona con los niveles del complejo PON1-HDL y proporciona una estimación muy precisa de la cantidad de partículas de HDL a las que está unido este enzima (84).

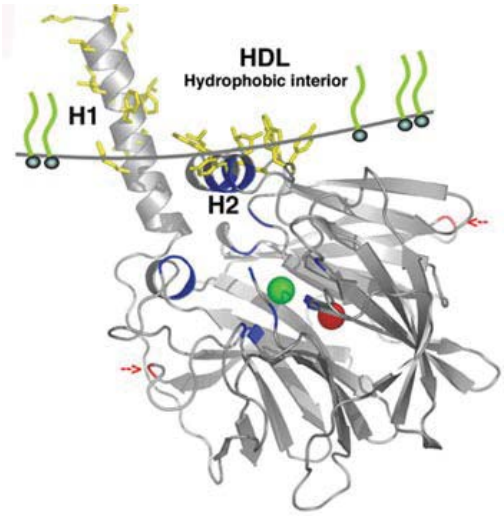

Figura 5: Estructura terciaria de PON1, modelo de anclaje de las partículas HDL. Figura adaptada de Harel M. et al (82).

Por último, existe otro método para la medición de la actividad lactonasa, éste utiliza como sustrato el 7-*O*-dietil fosforil 3-ciano 4-metil 7-hidroxicumarina (DEPCyMC) y permite una muy buena estimación de los niveles totales de PON1, tanto unida como no, a las HDL. La medición de la capacidad lactonasa mediante este método se realiza a 25ºC y a una longitud de onda de 400 nm.

Los métodos para la medición de la actividad lactonasa tienen dos ventajas respecto a los primeros, la primera es que la medición de la actividad de PON1 es más cercana a la actividad fisiológica de este enzima, si se realiza mediante los métodos que miden la actividad lactonasa, y la segunda y, no menos importante, es la ausencia de toxicidad.

La expresión de las actividades, bien sea paraoxonasa o bien sea lactonasa, se formula en U/L, siendo una unidad (U) equivalente a 1 µmol de paraoxon, TBBL o DEPCyMC, hidrolizado por minuto.

En los años 90 varios grupos generaron anticuerpos frente PON1 (85, 86) que fueron utilizados en el desarrollo de métodos de ELISA para la medida de la concentración de PON1 y para estudios inmunohistoquímicos de la expresión de este enzima (87). Uno de estos métodos utiliza un anticuerpo policlonal de conejo generado frente al péptido sintético CRNHQSSYQTRLNALREVQ, secuencia específica para PON1 (88).

Las actividades específicas de PON1 suelen expresarse como el ratio entre la actividad paraoxonasa o lactonasa respecto a la concentración de PON1 correspondiente, denominándose actividad específica paraoxonasa y actividad específica lactonasa respectivamente, las cuales, comúnmente se expresan en U/g.

4.2.3 Molécula ligando de quimiocinas 2

La molécula ligando de quimiocinas 2 (CCL2) es una quimiocina o citocina quimioatrayente, que forma parte de una familia de pequeñas proteínas de secreción con estructura globular que actúan como mensajeros para activar y dirigir la migración de leucocitos específicos del torrente sanguíneo hacia los tejidos lesionados. La CCL2, también

denominada MCP-1, juega un papel muy importante en la regulación de la respuesta inflamatoria (27, 89).

Las quimiocinas están asociadas a la migración de las células inmunes y, a su vez, también juegan un papel importante en el correcto funcionamiento de las rutas metabólicas.

La función más conocida de la CCL2 es la estimulación de la migración de los monocitos a los lugares inflamatorios y la unión con los receptores de unión de las quimiocinas de motivo CC (CCR2) que se expresan en la superficie celular y promueven el acumulo de monocitos en el endotelio vascular (90, 91).

Tanto la proteína CCL2 como su mRNA se expresan en la mayoría de los tejidos, lo cual sugiere que hay una producción sistémica y una respuesta al estímulo inflamatorio (92). Se ha observado que la síntesis de CCL2 se encuentra presente en aquellas enfermedades que presentan infiltrados ricos en monocitos, entre estas enfermedades se incluye la aterosclerosis, la insuficiencia cardíaca congestiva y la artritis reumatoide.

Muchos estímulos, particularmente el estrés oxidativo, causan un incremento de la producción de CCL2 por las células vasculares y un estímulo de la formación de células espumosas, inflamación y progresión del proceso aterosclerótico (90, 93). El incremento de los niveles de CCL2 también se ha observado en la EAP respecto a los sujetos sanos.

El estudio de la relación entre CCL2 y PON1 puede ayudar a entender la biopatología de la EAP ya que, por ejemplo, un incremento de la producción de CCL2 por las células endoteliales puede ser inhibido de una manera significativa por la acción de PON1 (91, 94).

Los estudios llevados a cabo por nuestro grupo han demostrado que la actividad y concentración sérica de PON1 son significativamente inferiores en los pacientes con EAP respecto a sujetos sanos y las concentraciones de CCL2 son significativamente superiores (92, 95, 96).

4.2.4 Proteínas carboniladas

El indicador más ampliamente utilizado para medir la oxidación de las proteínas es el contenido en proteínas carboniladas. La carbonilación representa la modificación por oxidación que se produce con más frecuencia en las proteínas (23).

Los cationes generados mediante el ciclo redox, tales como Fe^{2+} o Cu^{2+}, pueden unirse a los lugares de unión específicos para cationes que existen en la estructura de las proteínas, los cuales, cuando están unidos a H_2O_2 o O_2, pueden transformarse en carbonilos gracias a los grupos amino de diversos aminoácidos. La oxidación de las proteínas se cataliza mediante metales, pero no es el único mecanismo mediante el cual los carbonilos se introducen en la estructura proteica, por ejemplo, los aldehídos y el humo del tabaco también están implicados en la oxidación de proteínas.

Los métodos desarrollados para la cuantificación de la extensión de la carbonilación de proteínas son diversos. El método más comúnmente usado consiste en la derivatización de las proteínas carboniladas mediante la 2,4-difenilhidracina (DNPH) y su posterior medición mediante un ensayo espectrofotométrico. Este método permite cuantificar el contenido de proteínas carboniladas gracias a la capacidad que posee la DNPH para reaccionar con los carbonilos y producir un aducto que absorbe la luz a 366 nm, propiedad que se utiliza para medir su concentración mediante espectrofotometría. La cuantificación de las proteínas carboniladas mediante este método es costosa en tiempo y no es posible realizar una medición de alto rendimiento, por otra parte la cantidad de muestra con la que se trabaja es relativamente alta y existen pérdidas de proteínas al realizar los diferentes lavados con ácido (97).

Este método se ha mejorado al incluir la cromatografía líquida de alta resolución como método de separación y la detección paralela de aductos absorbentes; la ventaja principal de este método es la detección de las diferentes moléculas.

A su vez, con posterioridad, se han desarrollado técnicas inmunológicas de ELISA y Western Blot para incrementar la sensibilidad en la detección de proteínas carboniladas en las muestras (98).

En la actualidad estos métodos son ampliamente utilizados para evaluar los cambios en la cantidad de carbonilos respecto a las proteínas totales y, en algunos casos, más específicamente, evaluar qué proteínas concretamente han sido sometidas a oxidación, es para ello que la espectrometría de masas resulta muy útil gracias a su capacidad para identificar las proteínas carboniladas y los residuos aminoacídicos asociados que se han modificado a derivados de carbonilo.

Aunque estos métodos, debido a su gran potencial en la medición del estrés oxidativo, presentan la ventaja de su elevada precisión y especificidad respecto al primero, requieren de la utilización de equipos muy costosos, lo cual limita su utilización (99).

4.2.5 Galectina-3

Las galectinas son una familia de lectinas con una alta afinidad por los β-galactósidos; esta familia fue identificada tras el trabajo de Ashwel y Morell quienes, en la década de los 60, caracterizaron el receptor de las asialoglicoproteinas (100).

Esta familia de lectinas está altamente conservada en los mamíferos e íntimamente relacionada con la regulación de la inflamación. Las galectinas, a su vez, se clasifican en tres grupos distintos en función de su estructura cuaternaria: prototípica, quimérica y repetición en tándem (101). Todas las galectinas contienen en su estructura un dominio de reconocimiento de carbohidratos (CRD) en su extremo C-terminal, sin embargo la galectina-3 es el único miembro de la familia que contiene un CRD unido a un dominio N-terminal rico en prolina, glicina, alanina y tirosina (102).

La galectina-3 es una proteína de localización citoplasmática que puede ser secretada extracelularmente, esta es la propiedad que le permite unirse a los carbohidratos de la superficie celular, que contienen beta-galactósidos. Esta capacidad de unión a carbohidratos le confiere la propiedad de participar en una gran variedad de procesos biológicos incluidos la proliferación, quimiotaxis de macrófagos, fagocitosis, extravasación de neutrófilos, estrés oxidativo, apoptosis y angiogénesis.

Todos estos mecanismos están presentes en las enfermedades cardiovasculares (103).

La galectina-3 se expresa en condiciones normales en monocitos, eosinófilos y macrófagos, así como en una gran variedad de células epiteliales de diversos órganos (colon, mama, estomago, cerebro, ovario, hígado y riñón humano). En condiciones patológicas se encuentra suprarregulada y se expresa en linfomas, células T infectadas por HTLV-1, carcinoma de mama, carcinoma de cólon, hepatocarcinoma, melanoma y carcinoma tiroideo (104, 105).

El estudio de la galectina-3 en relación con el fallo cardíaco ha demostrado su aplicabilidad en esta patología, parece que la galectina-3 tiene un valor predictivo positivo en relación con la predicción de fallo cardíaco o infarto agudo de miocardio prematuro (106-111).

Debemos tener en cuenta que su implicación en la aterosclerosis todavía no ha quedado definida.

La galectina-3 juega un papel muy importante en la fibrosis vascular. Se ha observado que produce un incremento de la expresión de colágeno I en las células del músculo liso del endotelio vascular (112), y está involucrada en el proceso de conversión de monocitos a macrófagos gracias a sus efectos quimiotácticos (113). Su papel en la inflamación está relacionado con el reclutamiento de diferentes células tales como monocitos, eosinófilos, neutrófilos o linfocitos T (112). Por otra parte ya está demostrado el papel que juegan las galectinas en la angiogénesis (114); hay estudios que afirman y demuestran que la galectina-3 promueve la angiogénesis, este hecho se reveló en la investigación realizada con células endoteliales de venas umbilicales (115) y Markowska et al. en 2010 demostraban en sus estudios *in vivo* cómo la galectina-3, gracias a su capacidad de unión a carbohidratos, estimulaba la expresión del receptor del factor de crecimiento endotelial vascular (VEGF-r), promoviendo la fosforilación del mismo e incrementando la capacidad de unión con el propio factor de crecimiento endotelial vascular (VEGF), para, de este modo, acrecentar la síntesis vascular (116) (Figura 6).

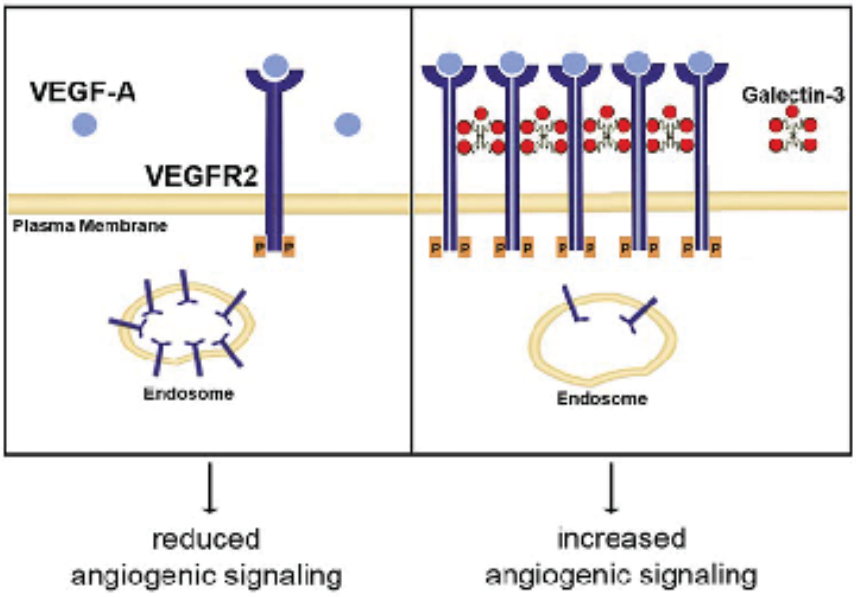

Figura 6: Estimulación de la expresión del VEGF-r mediada por la galectina-3 con . Figura adaptada de Markowska AI. et al (116).

Y finalmente se debe destacar la reciente demostración de la relación de la misma con los procesos de estrés oxidativo que se dan en la EAP, en los estudios publicados por Madrigal-Matute et al. en 2014 (101).

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HIPÓTESIS Y OBJETIVOS

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1. Hipótesis:

La EAP es una manifestación de la aterosclerosis que conlleva, por tanto, una desregulación entre los mecanismos pro-oxidantes y antioxidantes. Nuestra hipótesis es que mediante la investigación de parámetros bioquímicos relacionados con el estrés oxidativo y la inflamación, podremos encontrar biomarcadores eficaces para la evaluación y estudio clínico de esta enfermedad.

2. Objetivo:

Investigar la utilidad de marcadores emergentes relacionados con el estrés oxidativo y la inflamación en el estudio de la EAP.

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RESULTADOS

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ESTUDIO 1

Biochemical indices of oxidative stress and inflammation in the evaluation of peripheral artery disease

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ABSTRACT

Background: The aims of this study were: (1) to investigate changes in indices of oxidative stress and inflammation in the evaluation of peripheral artery disease (PAD); (2) to compare the diagnostic efficacy of these parameters with that of classical clinical laboratory routine parameters.

Design and methods: We studied 115 patients with PAD and 300 healthy volunteers.

Results: PAD patients had significantly increased circulating concentrations of F_2-isoprostanes, protein carbonyls, chemokine (C-C motif) ligand 2 (CCL2), high-sensitivity C-reactive protein (hs-CRP), β-2-microglobulin (B2M), and decreased paraoxonase-1 (PON1) levels. When patients were classified according to the Fontaine score, we observed important increases in plasma F_2-isoprostanes and CCL2 that appeared in milder stages of the disease, and remained so at similar levels in more advanced stages; almost no overlapping with the control group was noted. Receiver operating characteristics analysis comparing patients and controls revealed that the areas under the curve for F_2-isoprostanes and CCL2 approached unity [0.999 (0.998 – 1.000) and 0.993 (0.985 – 1.000), respectively, and significantly higher to those of the other measured parameters.

Conclusion: Our data suggest that F_2-isoprostanes and CCL2 measurements may be useful tools for the diagnosis of PAD.

Key words: chemokine (C-C motif) ligand 2; inflammation; F_2-isoprostanes; oxidative stress; paraoxonase-1; peripheral artery disease; protein carbonyls.

1. Introduction

Lower-extremity peripheral artery disease (PAD) is an important manifestation of atherosclerosis that is associated with severe impairment of different arterial territories. Indeed, PAD is a predictor of coronary and cerebral vascular disease risk [1]. The disease prevalence increases with age and, in people over the age of 70 years, it is estimated to be about 20% [2]. Inflammation is important for the initiation and progression of PAD, and the inflammatory mediators involved in this process are similar to those contributing to coronary artery disease. Smoking and diabetes mellitus, the strongest predictors of PAD, promote oxidative stress, which enhances inflammatory pathways [3]. Atherosclerosis affects wide portions of arteries in the lower extremities of PAD patients, resulting in progressive functional impairment and decline, and greatly reducing quality-of-life. This deterioration is the effect of a silent progression of the disease and, as such, appropriate and effective prevention measures are applied too late, or not implemented at all [2,4,5]. Moreover, asymptomatic PAD is several times more common in the general population than the symptomatic disease [6]. Hence, the search of biomarkers to enable early diagnosis of the disease, or the prediction of future complications, is an active line of research. Several studies have proposed C-reactive protein, measured by a high-sensitivity method (hs-CRP), or β-2-microglobulin (B2M) as useful markers of PAD. However, their use in the diagnosis of this disease is still an unresolved issue [7,8].

Oxidative stress is implicated in the development of atherosclerotic vascular diseases, including PAD [9]. Several studies have reported that oxidized low-density lipoprotein uptake by macrophages

promotes, among other responses, an inflammatory reaction that increases the production of the inflammatory chemokine (C-C motif) ligand 2 (CCL2). The consequence is the stimulation of arterial fatty streak formation and atheroma [10]. Oxidative stress can be evaluated in the circulation through the measurement of several markers such as lipid peroxidation, protein carbonylation or DNA damage. Isoprostanes are products of arachidonic acid and other polyunsaturated fatty acids. Similar to prostaglandins, they are generated *via* free-radical catalyzed mechanisms [11]. The subclass composed of F_2-isoprostanes, is one of the most abundant and, of these, 8-iso-prostaglandin $F_{2\alpha,}$ (8-iso-$PGF_{2\alpha}$) measurement is considered an accurate method for evaluating oxidative stress in humans [12]. PAD patients have increased serum 8-iso-$PGF_{2\alpha}$, and this parameter is seen as an independent predictor of the disease [13]. In contrast, data on oxidative damage to proteins are scarce. Among the numerous products of oxidative damage of proteins, measurement of protein carbonyls is the most widely used type of protein damage for inferring oxidative stress. Specific protein carbonylations are thought to have clinical significance, since they function as biological signals or of irreversibly altered protein structure and function [14]. Conversely, oxidative stress is counteracted by several antioxidant systems, one of the most important being the paraoxonase (PON) enzyme system. These antioxidant enzymes protect lipoproteins and cells from peroxidation and, as such, can mitigate the atherosclerosis process and vascular diseases [15]. The PON family contains three enzymes: PON1, PON2 and PON3, the genes of which are located adjacent to each other on chromosome 7q21-22. PON1 and PON3 are found in many tissues, as well as in blood, where they are associated with high-density lipoproteins (HDL). Conversely, PON2 is exclusively intracellular [15]. The most

extensively studied among the PON enzyme family is PON1 because alterations in the structure and concentration reflect pathological status. Also, it is the most abundant in the circulation and can be analyzed by simple laboratory methods. Clinical data suggest that circulating serum PON1 activity may be an important marker of a variety of diseases that involve an inflammatory response to an increased oxidative stress, including PAD [16-18].

The aims of the present study were: (1) to investigate changes in F_2-isoprostanes, protein carbonyls, PON1, and CCL2, in the progression of PAD; (2) to compare the diagnostic efficacy of these parameters with that of hs-CRP and B2M.

2. Materials and methods

2.1. Ethics approval

The Hospital's Ethics Committee (Institutional Review Board) approved the procedures of the study (approval documents 10-04-29/4proj3 and 2011-10-27/10proj1), and written informed consent was obtained from the participants.

2.2. Participants

This is a prospective, observational, cross-sectional study in patients regularly attending the Vascular Disease Department of Hospital Universitari Joan XXIII. Diagnosis of PAD was performed with measurements that included the ankle–brachial index (ABI), non-invasive imaging, and angiography when indicated. We recruited 115 patients; 45 having an ABI between 0.4 and 0.9, and 70 with an ABI lower than 0.4. The Fontaine classification was used to evaluate the degree of PAD

severity [19]. Participants with clinical or analytical evidences of infection, acute ischemia, renal failure, liver disease, cancer, or autoimmune diseases were excluded. Diabetes, hypertension and dyslipidemia were defined according to established criteria [20-22]. The control group was composed of 300 blood samples obtained from healthy volunteers participating in a population-based study. Participants of white-Mediterranean ethnic origin, were identified as being ostensibly healthy with no clinical or analytical evidence of infectious disease, renal insufficiency, hepatic damage, neoplasia, oligophrenia, or dementia. Thirty-three participants had hypertension (11.0%), 16 had dyslipemia (5.3%), and 11 had type II diabetes mellitus (3.7%). Medication intake was not an exclusion criterion except in the case of drugs interfering with vitamin metabolism (methotrexate, tuberculostatics, theophylline, or vitamin B_6 antagonists). The population studied did not consume vitamin supplements or local food fortified with vitamins. Pregnant women were not included in the study. The samples were stored at –80ºC in our Biological Sample Bank. A detailed description of this population has been published previously [23].

2.3. Biochemical measurements

The true physiological substrates for PON1 have not as yet been identified. Since PON1 has lactonase and esterase activities [15], we opted to analyze the catalytic activity of PON1 using two different substrates: 5-thiobutyl butyrolactone (TBBL, a synthetic lactone) and paraoxon (an ester), as previously described [16]. Briefly, TBBLase activity was measured in an assay containing 1 mM $CaCl_2$, 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 (1 U = 1 mmol of TBBL hydrolyzed per minute). Serum PON1 paraoxonase activity was determined as the rate of hydrolysis of paraoxon at 410 nm and 37ºC in a 0.05 mM glycine buffer, pH 10.5 with 1 mM $CaCl_2$ [24] . Activities were expressed as U/L (1 U = 1 μmol of paraoxon hydrolyzed per minute). Serum PON1 concentrations were determined by an in-house enzyme-linked immunosorbent assay (ELISA) with a rabbit polyclonal antibody generated against the synthetic peptide CRNHQSSYQTRLNALREVQ which is sequence specific for mature PON1 [25]. PON1 specific activities were calculated as the ratio between the activity and the corresponding concentration. The ethylene diamine tetraacetate (EDTA)-plasma concentrations of F_2-isoprostanes were determined by Enzyme Immunoassay (Cayman Chemical Co., Ann Arbor, Michigan, USA). Plasma protein carbonyl content was measured by a colorimetric assay (Cayman Chemical Co.). The hs-CRP concentration in serum was measured by automated turbidimetry (Roche Diagnostics, Mannheim, Germany). The serum concentrations of B2M and the EDTA-plasma concentration of CCL2 were measured by ELISA (Biovendor, Brno, Czech Republic, Roche Diagnostics, and Prepotech, London, UK, respectively). Serum cholesterol, HDL cholesterol, triglycerides, and insulin concentrations were analyzed by standard automated procedures in the Clinical Chemistry laboratory of *Hospital Universitari Joan XXIII*.

2.4. Statistical analyses

All calculations were performed with the statistical package for social sciences (SPSS 22.0, Chicago, IL, USA). Differences between any two groups were assessed with the χ^2 test (categorical) or the Mann-Whitney *U* test (continuous), since most of the studied variables had

non-parametric distributions. Differences between more than two groups were assessed by the Kruskal-Wallis test. Spearman correlation coefficient was used to evaluate the degree of association between variables. The diagnostic accuracy of the measured biochemical variables was assessed by receiver operating characteristics (ROC) curves [26]. A binary logistic regression analysis using the Wald forward step-by-step method was applied to identify independent predictors of PAD. The stepwise selection method is one in which the test-for-entry is based on the significance of the score statistics, and the test-for-removal is based on the lack of significance of the association between the putative predictor and the dependent variable. Candidate variables selected for logistic regression modeling were age, gender, smoking status, hypertension, diabetes mellitus, dyslipidemia, hs-CRP, B2M, F_2-isoprostanes, lactonase activity, PON1 concentration, and CCL2. Wald statistics were reported for variables in the final model. Unless otherwise indicated, results are shown as medians and 95% Confidence Interval (CI).

3. Results

3.1. Clinical characteristics of the PAD patients

A large majority of the patients were symptomatic, according to the Fontaine classification and most were at stages III and IV when recruited into the study. As expected, patients were older and the percentage of men was higher than in the control group. PAD patients were in receipt of several medications for the treatment of their disease. Intake of vitamins or mineral supplements was not recorded. The incidence of current habit of smoking and alcohol consumption was

lower in the patients reflecting, perhaps, adherence to their physician's recommendations. Hypertension, diabetes mellitus, dyslipidemia, heart disease and pulmonary disease were common in PAD patients, but cerebrovascular events were not recorded (Table 1).

3.2. Selected biochemical variables are altered in PAD patients but they are not strongly related to the severity of the disease

Compared to the control group, PAD patients had significant increases in the circulating concentrations of triglycerides, insulin, hs-CRP, B2M, F_2-isoprostanes, protein carbonyls, and CCL2, together with decreases in PON1 lactonase and paraoxonase activities and PON1 concentrations. Since the decrease in serum PON1 concentration was higher than that of the enzyme activities, both lactonase and paraoxonase specific activities were significantly increased in patients with PAD (Table 1). When patients were classified according to the Fontaine score, we observed important increases in F_2-isoprostanes and CCL2 that appeared in milder stages of the disease, and remained at similar levels in more advanced stages. There was almost no overlap with the control group. Serum PON1 concentration and PON1 lactonase and paraoxonase activities showed a progressive decrease, albeit the degree of overlap was higher. Protein carbonyls and the standard tests (hs-CRP and B2M) significantly increased in relation to the Fontaine score but, in general, alterations appeared later to those F_2-isoprostanes or CCL2, and the degree of overlap among groups was considerable (Figures 1 and 2).

As expected, we observed significant direct correlations between markers of oxidative stress and inflammation in PAD patients, and

inverse correlations between these markers and PON1-related variables (Figures 3 and 4).

Because the PAD patients and the control group were clearly different in terms of age and gender distribution, we investigated the influence of these characteristics on the analyzed parameters. Results are shown in Supplementary Tables 1 and 2. Among the investigated parameters none was influenced by gender, neither in the control group nor in the PAD patients. Only paraoxonase and lactonase activities were slightly influenced by age in the control group. Raw data for the PON-1 related variables and an example of the calibration curves for serum PON1 concentration measurements are shown in Supplementary Table 3 and Supplementary Figure 1, respectively. Intake of medications did not produce any significant change in F_2-isoprostanes concentrations in PAD patients (Supplementary Table 4).

3.3. Diagnostic accuracy of the selected biochemical variables

When comparing the diagnostic accuracy of the selected variables in discriminating between the healthy volunteers and the patients with PAD, we found that the AUROC for F_2-isoprostanes and CCL2 were very close to unity, and significantly higher to those of the other measured parameters. The order of the calculated accuracies was: F_2-isoprostanes ≈ CCL2 > PON1 concentration > B2M > lactonase ≈ hs-CRP > paraoxonase specific activity ≈ lactonase specific activity ≈ protein carbonyls ≈ paraoxonase (Figure 5).

3.4. Binary logistic regression analysis

Wald's forward binary regression analysis identified, in step 1, F_2-isoprostanes as the only biochemical variable significantly associated with PAD. Step 2 introduced CCL2, although the P value did not reach statistical significance. All the other investigated variables (demographical and clinical variables, PON1 concentration, B2M, lactonase, and hs-CRP) did not enter into the model (Table 2).

4. Discussion

According to current consensus, atherosclerosis represents a state of enhanced oxidative stress and inflammation characterized by lipid and protein oxidation in the vascular wall, together with increased synthesis of pro-inflammatory cytokines and chemokines. The oxidative modification hypothesis of atherosclerosis predicts that low-density lipoprotein oxidation is an early event in atherosclerosis and that oxidized low-density lipoproteins plays a key role in the onset and development of this disease. Changes include the production of reactive oxygen and nitrogen species by vascular cells, as well as oxidative modifications contributing to important clinical manifestations of artery disease, such as endothelial dysfunction [27]. Oxidative stress and inflammation are phenomena that have been extensively investigated in relation to coronary artery disease and myocardial infarction. However, its relationship with PAD is not as well documented. This is surprising since the clinical burden of atherosclerosis is much higher in PAD than in coronary disease which, *a priori*, suggests that alterations in the circulation are much higher and would correlate better with the disease.

Our findings are in accordance with previous studies documenting enhanced oxidative stress in patients with PAD [13,28-30].

The F_2-isoprostanes have been reported to be the "gold standard" to assess oxidative injury *in vitro* [12]. Our patients had, on average, 10 times higher plasma concentrations of F_2-isoprostanes than the control group. These results were observed from the earliest stages of the disease (Fontaine stages I-II). An increase in F_2-isoprostanes in PAD patients had been reported 11 years ago by Mueller et al. [13] i.e. that 8-iso-$PGF_{2\alpha}$ was an independent predictor of PAD. However, despite their important findings, measurement of 8-iso-$PGF_{2\alpha}$ as a clinically-relevant biomarker of the disease has not been implemented, probably because the reported differences with their healthy population were lower than those observed, for example, in the present investigation. Indeed, we obtained ROC curves with an excellent clinical sensitivity and specificity, and multivariate analysis retained only F_2-isoprostanes as an independent predictor of PAD, while excluding hs-CRP, B2M and all the other measured variables. Very recent studies also found increased plasma concentrations of isoprostanes in PAD patients. Berent et al. [31] reported increased levels of 8-epi-prostaglandin $F_2\alpha$ in 100 patients with PAD, and Loffredo et al. [32] observed high levels of serum prostaglandin $F_2\alpha$III in PAD patients at Fontaine stage IIb, levels that decreased after consumption of dark chocolate rich in antioxidant polyphenols. There is only one study reporting contradictory findings. Signorelli et al. [33] found decreased levels of F_2-isoprostanes in a small group of patients with an ABI ≤0.9 together with significant increases in microRNAs (miR) miR-130, miR-210 and miR-27b. The authors interpreted these findings as being an adaptive response in these patients to reduce oxidative stress (i.e., antioxidant enzymes upregulation).

All these studies, including our own, measured isoprostanes by ELISA. This may represent a problem, since ELISA assays are less accurate than gas chromatography/mass spectrometry (GC/MS) due, mainly, to cross reactivity [34]. There has been a dearth of follow-up studies comparing ELISA *versus* GC/MS in PAD. Unfortunately, GC/MS methods are expensive, are not always feasible, and are difficult to implement as routine clinical chemistry tests. Indeed, and apart from any considerations on the precise nature of the molecules measured by the ELISA method, our results showed that the measurement of F_2-isoprostanes (or "F_2-isoprostanes-like substances") by ELISA almost perfectly discriminates between the healthy population and the PAD patients. As such, it is an excellent biomarker of this disease.

Our study confirms the reported increase in plasma CCL2 concentration and decrease in serum PON1 activities and concentrations in PAD [18,35]. We have previously reported that the ratio between CCl2 and PON1-related variables segregated controls from patients almost perfectly [18]. We showed that the inflammatory response, probably secondary to enhanced oxidative stress, is extreme in PAD patients suggesting that this is causally related to the progression of the disease. A possible explanation for serum PON1 activity being decreased in PAD patients is that PON1 is inactivated by oxidized lipids. This has been demonstrated by studies demonstrating that the incubation of PON1 *in vitro* with oxidized palmitoyl arachidonoyl phosphatidylcholine, lysophosphatidylcholine, and oxidized cholesteryl arachidonate, results in the negation of PON1 activity [15]. We reasoned that patients with PAD represent a clinical model with considerable multiple vessel disease and high burden of atherosclerosis. We found that results obtained from the

measurement of plasma CCL2 and serum PON1 concentrations, as well as PON1 paraoxonase and lactonase activities, confirm our hypothesis, and may constitute novel indicators of disease status. In our study, decreased PON1 activities were associated with increased concentrations of CCL2. However, this inverse relationship was not confirmed at tissue level. Indeed, previous studies from our group showed that the expression of both proteins is increased in the arteries of patients with PAD [36]. This observation would suggest that the variations in serum PON1 and plasma CCL2 concentrations do not necessarily correlate with their putative roles at the cellular level. Perhaps PON1 protein expression is increased in diseased arteries to counteract oxidative stress and CCL2-induced inflammation. However, this hypothesis needs to be confirmed by further investigations. An interesting result from our study was to observe an increase in both paraoxonase and lactonase specific activities in PAD patients, due to the decrease in PON1 concentration being higher than that of the enzyme activities. An explanation for this phenomenon cannot be ascertained from the present investigation; albeit we have already observed discrepancies between changes in PON1 activity and its concentration in other non-communicable diseases [37,38]. PON1 activity is strongly dependent on the HDL structure and composition [39,40] and, perhaps, the increased lipid/PON1 protein ratio in the HDL particles of PAD patients increases the enzyme activity as a compensatory mechanism. Currently, however, we can only speculate, and further studies are needed to explore this hypothesis.

In contrast, oxidative modification of proteins does not seem to be important in our patients, since the increase of protein carbonyls is not as marked as that of F_2-isoprostanes. This is consistent with the data

contained in previous reports [41,42]. A possible explanation for this relatively small increase in protein carbonyls may lie in the observation that carbonylated proteins have an accelerated turnover since damaged proteins activate proteosomes for their degradation. Thus, carbonylated proteins enter the degradation pathway rather than the chaperone/repair pathway i.e. carbonylation represents an irreversible, and non-repairable, modification [43,44].

The results from the present investigation suggest that F_2-isoprostanes and CCL2 are superior to hs-CRP and B2M in the diagnosis of PAD, especially at the earliest stages of the disease. Several studies reported that serum hs-CRP concentrations are elevated in PAD patients, and that the values correlated with ABI and endothelial function [7,45,46]. More recent studies identified B2M protein concentrations as being higher in PAD [47,48]. However, the reported elevations were moderate and, hence, panels of several biomarkers were proposed to enhance their diagnostic accuracy. One of these panels included cystatin C, hs-CRP, B2M, and glucose but, despite this recommendation, the AUROC was no higher than 0.70 [49]. Another study proposed the inclusion of the genetic marker the rs10757269; a polymorphism at the chromosome 9p21 locus. When added to B2M, cystatin C, and hs-CRP, the discriminatory power of the panel was increased considerably. However, the main drawback of this proposal is that genetic markers may be influenced by ethnicity, and this issue has not been sufficiently investigated in the case of rs10757269 polymorphism [50]. Another proposed panel included 9 different biomarkers: hs-CRP, interleukin-6, tumor necrosis factor receptor-II, lipoprotein(a), N-terminal pro-brain natriuretic peptide, pro-atrial natriuretic peptide, C-terminal pro-arginine

vasopressin, osteoprotegerin, and fibrinogen [51]. These multiple panels are costly and difficult to implement in standard clinical practice. Conversely, we suggest that the measurement of a single parameter, F_2-isoprostanes or CCl2, does indeed segregate, almost perfectly, PAD patients from the healthy population. A caveat to the present study is that, being based on a Hospital setting, most of the patients studied are already clinically symptomatic. No doubt a useful biomarker would be that which is able to predict asymptomatic PAD. We found that alterations in the circulating levels of F_2-isoprostanes and CCL2 are considerably higher than those observed for the other parameters we had investigated, and appear earlier-on in the evolution of the disease. Thus, these levels may reflect early stages of the disease and, as such, would be invaluable in discriminating disease from non-disease status. We think that population-based studies in primary health centers (PHC) would be worth implementing to fully ascertain the utility of F_2-isoprostanes and CCL2 in the diagnosis of PAD, since the PHC represents the front-line in the discrimination of disease *vs*. non-disease status. Another question that emerges from our study is whether the intake of certain medications influences the concentrations of plasma F_2-isoprostanes. We had not recorded this aspect in the control group and, as such, we cannot be categorical on this issue. However, the finding that F_2-isoprostanes were already elevated in asymptomatic (or nearly asymptomatic) patients, added to the observation that there were no significant difference in F_2-isoprostanes levels segregated with respect to any particular medication, would suggest that the influence of this variable was minimal, if at all.

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Conflict of interest

None

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Figure legends

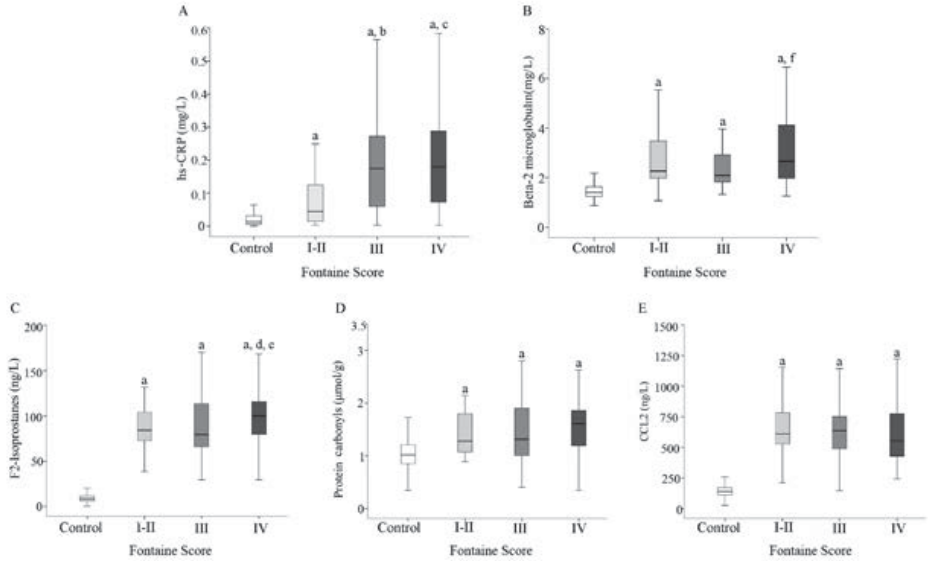


Fig. 1: Selected markers of oxidative stress and inflammation in PAD patients (classified according to the Fontaine score) and in the control group. Significance values by the Mann-Whitney *U* test: [a] $p < 0.001$, and [b] $p < 0.01$ with respect to the control group; [c] $p < 0.001$, and [d] $p < 0.05$ with respect to Fontaine stage I-II; [e] $p < 0.01$, and [f] $p < 0.05$ with respect to Fontaine stage III. Overall variations $p < 0.001$ by the Kruskal-Wallis test.

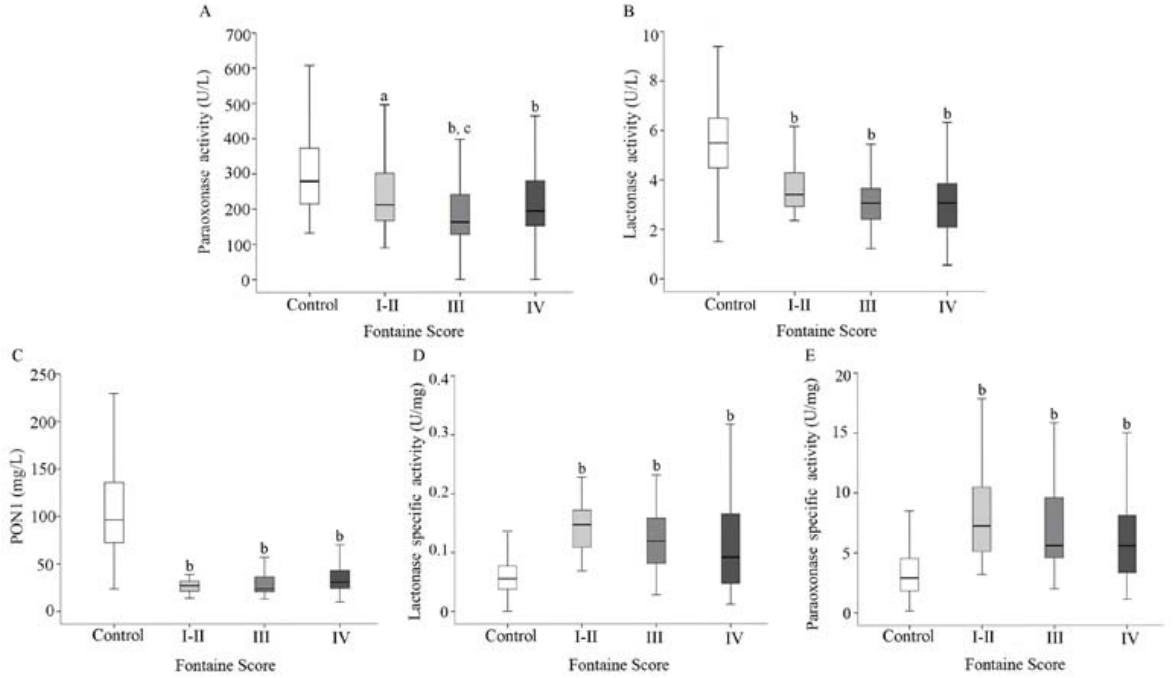


Fig.2: PON1-related variables in PAD patients (classified according to the Fontaine score) and in the control group. Significance values by the Mann-Whitney *U* test: [a] $p < 0.01$, and [b] $p < 0.001$ with respect to the control group; [c] $p < 0.01$ with respect to Fontaine stage I-II. Overall variations $p < 0.001$ by the Kruskal-Wallis test.

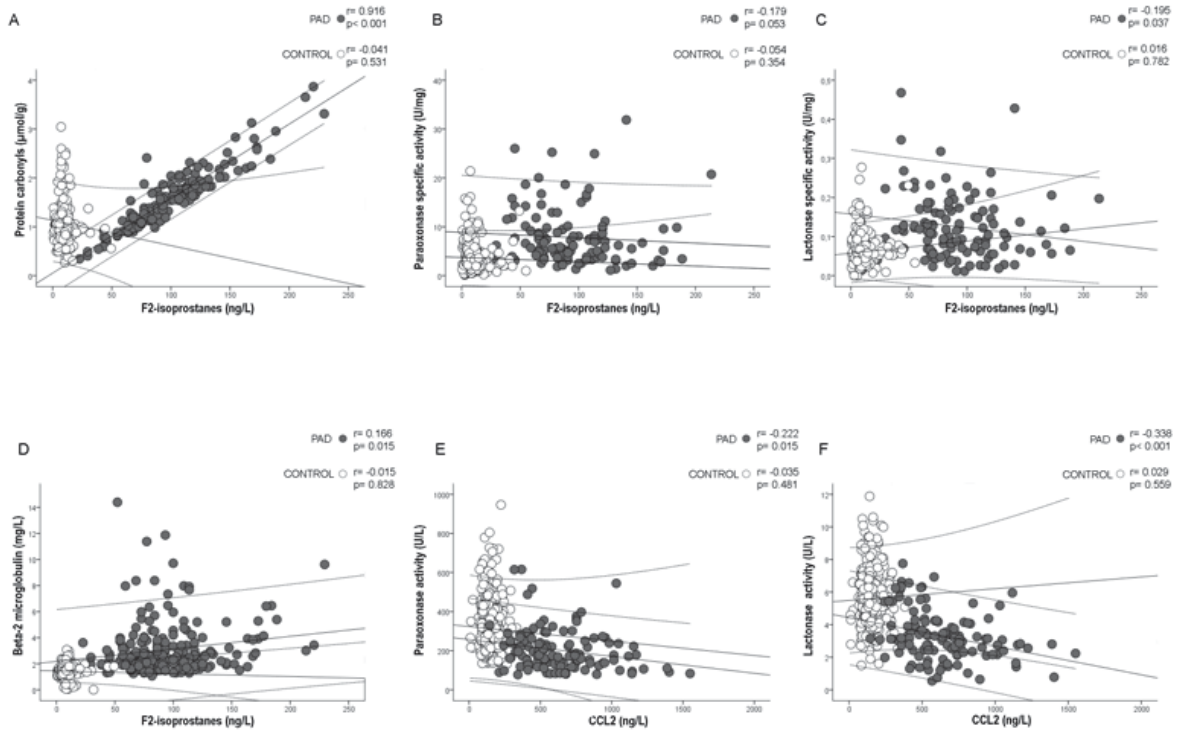


Fig. 3: Statistically significant correlations between selected biochemical variables. The continuous lines represent the regression lines, and the dashed lines, the 95% Confidence Intervals. Correlations calculated by the Spearman's ρ test.

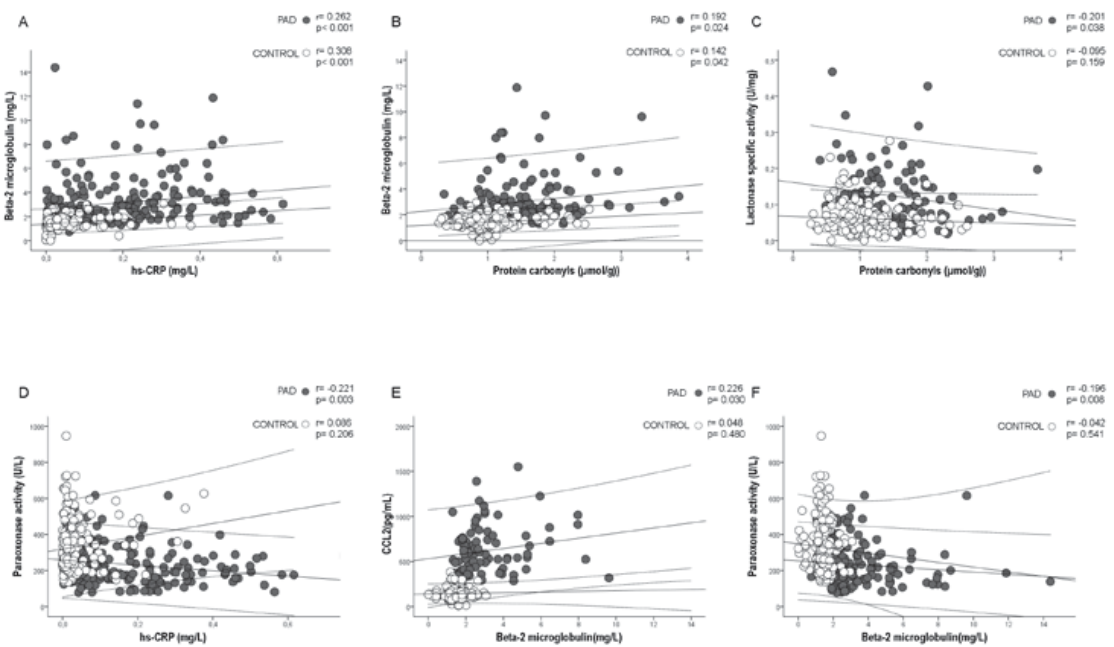
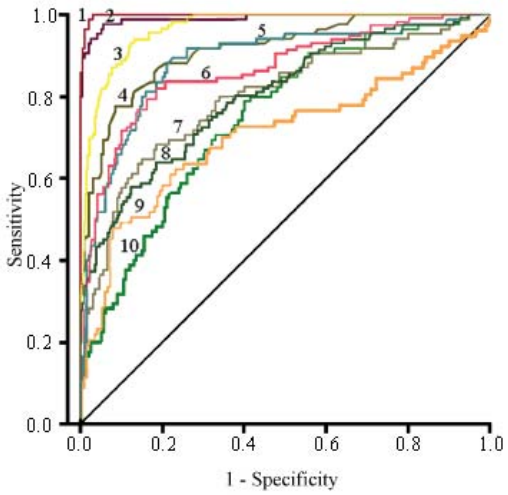


Fig. 4: Statistically significant correlations between selected biochemical variables. The continuous lines represent the regression lines, and the dashed lines, the 95% Confidence Intervals. Correlations calculated by the Spearman's ρ test.



	Variable	AUROC	SE	95% CI
1	8-isoprostanes	0.999	0.001	0.998 – 1.000
2	CCL2	0.993	0.004	0.985 – 1.000
3	PON1 concentration	0.966	0.007	0.952 – 0.980
4	β-2-microglobulin	0.899	0.015	0.871 – 0.928
5	Lactonase	0.856	0.022	0.813 – 0.899
6	hs-CRP	0.872	0.017	0.839 – 0.905
7	Paraoxonase specific activity	0.782	0.024	0.736 –0.828
8	Lactonase specific activity	0.780	0.028	0.726 – 0.834
9	Protein carbonyls	0.756	0.025	0.707 – 0.804
10	Paraoxonase	0.717	0.022	0.673 – 0.761

Fig. 5: Receiver operating characteristics (ROC) plots for all the studied biomarkers in PAD patients, and in the control group.

AUROC: areas under the curve of the ROC plots. SE: Standard Error.

TABLE 1: Demographic, clinical, and biochemical characteristics of PAD patients and the control group

Variable	Control group $n = 300$	PAD $n = 115$	p
Age, years	47 (18 – 80)	70 (50 – 92)	<0.001
Male gender, *N* (%)	130 (43.3)	100 (86.9)	<0.001
Smokers, *N* (%)	99 (33.0)	10 (8.7)	<0.001
Alcohol consumption > 20g/day, *N* (%)	75 (25.0)	8 (6.9)	<0.001
Arterial hypertension, *N* (%)	31 (10.3)	79 (68.7)	<0.001
Diabetes mellitus, *N* (%)	12 (4.0)	76 (66.1)	<0.001
Dyslipidemia, *N* (%)	12 (4.0)	46 (40.0)	<0.001
Ischemic heart disease, *N* (%)	0	28.6	<0.001
Chronic obstructive pulmonary disease, *N* (%)	0	19.5	<0.001
Fontaine classification score, *N* (%)			
Stage I, asymptomatic		3 (2.6)	
Stage II, intermittent claudication	N.A.	19 (16.5)	
Stage III, resting pain		27 (23.4)	
Stage IV, ulceration or gangrene		66 (57.4)	
Medications, *N* (%):			
Antiplatelet drugs		34 (29.6)	
Statins		64 (55.6)	
Antidiabetic drugs		56 (48.7)	
Angiotensin converting enzyme inhibitors		56 (48.7)	
Angiotensin receptor antagonists		55 (47.8)	
Calcium receptor antagonists	N.R.	74 (64.3)	
Diuretics		61 (53.0)	
Anti-arrhythmia drugs		98 (85.2)	
Beta-blockers		97 (84.3)	
Bronchodilators		79 (68.7)	
Cholesterol, mmol/L	5.2 (3.7 – 7.0)	3.9 (2.6 – 5.9)	<0.001
HDL cholesterol, mmol/L	1.46 (0.96 – 2.20)	1.06 (0.59 – 1.79)	<0.001
Triglycerides, mmol/L	1.1 (0.5 – 2.9)	2.9 (1.7 – 4.8)	<0.001
Insulin, µmol/L	49.4 (20.1 – 125.3)	78.9 (15.1 – 423.7)	<0.001
hs-CRP, mg/L	0.13 (0.01 – 1.42)	1.24 (0.09 – 4.67)	<0.001
β-2-microglobulin, mg/L	1.40 (0.44 – 2.29)	2.52 (1.34 – 8.63)	<0.001
F_2-isoprostanes, ng/L	8.59 (2.44 – 21.79)	90.91 (45.45 – 171.70)	<0.001
Protein carbonyls, µmol/g	1.02 (0.54 – 2.00)	1.53 (0.74 – 2.82)	<0.001
Lactonase, U/L	5.49 (3.20 – 8.84)	3.12 (1.17 – 6.27)	<0.001
Paraoxonase, U/L	278.77 (161.30 – 579.90)	197.17 (84.07 – 473.19)	<0.001
PON1 concentration, mg/L	96.46 (43.60 – 290.49)	27.10 (13.69 – 63.41)	<0.001
Lactonase specific activity, U/mg	0.055 (0.016 – 0.133)	0.114 (0.026 – 0.264)	<0.001
Paraoxonase specific activity, U/mg	2.90 (0.78 – 9.52)	5.82 (1.95 – 19.00)	<0.001
CCL2, ng/L	138.52 (85.55 – 258.96)	586.50 (284.75 – 1185.00)	<0.001

Except when otherwise indicated, results are shown as medians and 95% CI (in parenthesis).

CCL2: Chemokine (C-C motif) ligand 2; N.A.: Not applicable; N.R: Not recorded; PON1: Paraoxonase-1.

TABLE 2: Binary logistic regression analysis of the variables associated with the presence of PAD

		B	SE	Wald	*p*	Exp(B)	95% CI Exp(B)
Step 1	F_2-isoprostanes	0.211	0.039	29.6	<0.001	1.235	1.145 – 1.333
	Constant	-7.384	1.256	34.59	<0.001	0.001	
Step 2	CCL2	0.034	0.022	2.419	0.120	1.034	0.991 – 1.079
	F_2-isoprostanes	0.163	0.083	3.894	0.048	1.177	1.001 – 1.385
	Constant	-17.087	9.048	3.567	0.059	0.000	

Step 1: Cox and Snell r^2 = 0.668; Negelkerke r^2 = 0.957; −2log of likelihood: 28.379

Step 2: Cox and Snell r^2 = 0.693; Negelkerke r^2 = 0.992; −2log of likelihood: 5.575

All the other variables had been excluded from the model.

SUPPLEMENTARY TABLE 1: Influence of gender on the selected biochemical variables in patients with peripheral artery disease (PAD) and in the control group.

*Differences between men and women assessed by the Mann-Whitney *U* test. There were no significant differences in any of the analyzed parameters with respect to gender.

s.a.: specific activity

	Control group			PAD		
Parameter	**Men**	**Women**	***p****	**Men**	**Women**	***p****
F_2-isoprostanes, ng/L	8.4 (3.4 – 33.2)	8.8 (2.0 – 21.2)	0.909	88.6 (45.4 – 168.4)	100.0 (45.4 – 193.6)	0.548
Protein carbonyls, µmol/g	1.1 (0.6 – 2.4)	1.0 (0.5 – 1.9)	0.115	1.5 (0.8 – 2.6)	1.5 (0.7 – 5.1)	0.992
CCL2, ng/L	134.8 (88.4 – 230.1)	140.6 (83.1 – 272.2)	0.459	586.5 (286.8 – 1157.5)	574.5 (272.0 – 2450.0)	0.832
PON1, mg/L	93.6 (44.3 – 288.2)	98.2 (43.7 – 301.1)	0.183	27.1 (13.8 – 66.1)	27.6 (12.5 – 59.1)	0.980
Paraoxonase, U/L	279.0 (163.9 – 615.3)	274.6 (156.4 – 566.1)	0.678	193.8 (85.9 – 465.5)	225.7 (31.0 – 525.2)	0.142
Lactonase, U/L	5.5 (3.3 – 8.8)	5.5 (3.1 – 8.9)	0.959	3.1 (1.2 – 6-2)	3.5 (0.7 – 6.6)	0.074
Paraoxonase s.a., U/mg	3.0 (0.8 – 9.6)	2.8 (0.8 – 8.9)	0.224	5.6 (1.9 – 18.7)	7.1 (1.8 – 20.7)	0.308
Lactonase s.a., U/mg	0.06 (0.01 – 0.12)	0.05 (0.02 – 0.13)	0.163	0.11 (0.03 – 0.26)	0.13 (0.05 – 0.13)	0.293
hs-CRP, mg/L	0.15 (0.01 – 1.65)	0.12 (0.02 – 1.40)	0.726	1.28 (0.09 – 4.67)	0.95 (0.18 – 5.18)	0.947
β-2-microglobulin, mg/L	1.42 (0.23 – 1.93)	1.38 (1.01 – 2.35)	0.793	2.51 (1.37 – 9.64)	2.71 (1.31 – 5.27)	0.503

SUPPLEMENTARY TABLE 2: Influence of age on the selected biochemical variables in patients with peripheral artery disease (PAD) and in the control group.

*Differences between the different age ranges assessed by the Kruskal-Wallis test in the control group and by the Mann-Whitney *U* test in PAD patients. There were no PAD patients younger than 31 years of age. Only age was associated with changes in PON1-related variables in the control group.

s.a.: specific activity; y.o.: years old.

	Control group				PAD		
Parameter	**≤ 30 y.o.**	**31 – 50 y.o.**	**≥ 51 y.o.**	***p****	**31 – 50 y.o.**	**≥ 51 y.o.**	***p****
F_2-isoprostanes, ng/L	8.5 (3.4 – 34.1)	9.0 (1.7 – 20.2)	8.4 (2.7 – 23.2)	0.721	84.1 (70.5 – 140.9)	90.9 (45.4 – 172.6)	0.670
Protein carbonyls, μmol/g	1.0 (0.5 – 2.6)	1.0 (0.5 – 1.8)	1.0 (0.6 – 2.2)	0.831	1.3 (0.9 – 2.0)	1.5 (0.7 – 2.8)	0.395
CCL2, ng/L	140.5 (84.3 – 227.2)	142.3 (86.4 – 269.2)	134.9 (82,5 – 279.4)	0.225	673.4 (285.6 – 1015.3)	586.5 (284.7 – 1185.0)	0.967
PON1, mg/L	87.8 (50.5 – 237.3)	100.1 (45.8 – 332.2)	97.6 (38.9 – 285.3)	0.179	32.8 (12.4 – 70.2)	27.1 (13.7 – 63.4)	0.549
Paraoxonase, U/L	273.8 (152.5 – 621.8)	259.8 (154.3 – 582.8)	306.3 (172.9 – 566.9)	0.009	258.9 (151.8 – 487.6)	196.5 (84.0 – 465.2)	0.232
Lactonase, U/L	5.1 (3.2 – 8.2)	5.5 (3.0 – 8-0)	5.7 (3.3 – 9.0)	0.040	3.0 (1.2 – 6.6)	3.1 (1.2 – 6.3)	0.926
Paraoxonase s.a., U/mg	3.2 (1.0 – 9.8)	2.5 (0.6 – 8.2)	3.2 (0.9 – 10.6)	0.023	8.4 (2.2 – 31.9)	5.8 (2.0 – 19.0)	0.301
Lactonase s.a., U/mg	0.06 (0.02 – 0.11)	0.05 (0.01 – 0.11)	0.06 (0.02 – 0.17)	0.110	0.09 (0.02 – 0.42)	0.11 (0.03 – 0.26)	0.341
hs-CRP, mg/L	0.09 (0.01 – 0.13)	0.11 (0.01 – 2.0)	0.15 (0.01 – 1.4)	0.147	0.08 (0.04 – 0.52)	0.13 (0.01 – 0.47)	0.335
β-2-microglobulin, mg/L	1.30 (0.23 – 1.79)	1.36 (0.44 – 1.90)	1.44 (0.45 – 2.35)	0.017	2.23 (1.83 – 6.47)	2.52 (1.34 – 8.89)	0.854

SUPPLEMENTARY TABLE 3: Raw data for F_2-isoprostanes, CCL2 and PON1-related variables.

0: Control group; 1: PAD patients.

Note: this table is a copy of the original in the Spanish version of Excel® program, in which decimal numbers are separated from the integer by a comma, not by a decimal point.

Group	Isoprostanes (ng/L)	CCL2 (ng/L)	PON1 (mg/L)	Paraoxonase (U/L)	Lactonase (U/L)	Paraoxonase specific activity (U/mg)	Lactonase specific activity (U/mg)
0	7,2642	137,3	83,07	165,11	5,09	1,988	0,061
0	37,1594	137,61	117,84	251,83	6,39	2,137	0,054
0	17,043	288,52	340,49	411,56	7,09	1,209	0,021
0	6,4261	260,64	88,38	213,61	5,79	2,417	0,066
0	1,9558	147,61	88,38	173,99	4,99	1,969	0,056
0	15,3667	228,82	128,46	268,72	5,99	2,092	0,047
0	55,0406	139,12	150,52	149,54	4,99	0,993	0,033
0	10,617	140,03	173,86	253,23	5,59	1,457	0,032
0	0,8382	295,48	88,54	206,11	7,19	2,328	0,081
0	6,9848	85,48	217,11	263,91	6,69	1,216	0,031
0	17,8812	118,82	121,22	171,48	6,29	1,415	0,052
0	21,7927	129,73	182,87	138,52	3,39	0,757	0,019
0	2,7939	168,21	1410,21	182,18	7,89	0,129	0,006
0	13,9697	154,27	252,27	330,32	8,89	1,309	0,035
0	36,0418	160,33	103,03	212,93	5,39	2,067	0,052
0	14,2491	138,52	131,52	236,74	5,99	1,8	0,046
0	41,0709	154,58	79,36	261,71	6,19	3,298	0,078
0	18,7194	94,27			6,59		
0	19,837	163,36	134,9	163,27	5,39	1,21	0,04
0	3,6321	143,67	254,33	335,9	6,59	1,321	0,026
0	4,4703	238,52	121,22	167,23	5,29	1,38	0,044
0	1,9558	232,76	132,81	161,22	5,69	1,214	0,043
0	12,5727	123,67	148,58	148,88	4,79	1,002	0,032
0	15,9255	161,24	146,81	254,64	5,79	1,734	0,039
0	11,7345	85,79	95,46	345,16	7,39	3,616	0,077
0	3,0733	164,58	165,81	468,01	10,38	2,823	0,063
0	12,5727	161,24	54,89	195,65	5,69	3,564	0,104
0	10,8964	157,61	89,99	278,82	6,49	3,098	0,072
0	19,5576	151,55	85,16	211,71	7,89	2,486	0,093
0	32,6891	176,09	113,07	282,78	5,99	2,501	0,053
0	34,0861	104,88	51,35	143,44	4,29	2,793	0,084
0	5,0291	150,64	87,13	201,58	4,49	2,314	0,052
0	43,3061	97,61	94,39	183,76	5,29	1,947	0,056
0	15,3667	167,3	83,79	171,1	4,79	2,042	0,057
0	23,7485	138,21	78,21	304,57	7,19	3,894	0,092
0	5,0291	186,7	100,31	296,95	4,99	2,96	0,05
0	4,5	150,64	153,86	273,05	5,79	1,775	0,038
0	7,5	119,42	92,38	175,47	5,29	1,899	0,057
0	7,8	267,61	95,97	185,88	5,49	1,937	0,057
0	3,9	173,06	162,53	179,99	4,7	1,107	0,029
0	5,7	141,55	82,13	220,45	6,89	2,684	0,084
0	4,5	140,64	113,49	402,23	5,99	3,544	0,053
0	8,7	147,3	109,07	340,04	4,2	3,118	0,039
0	4,5	120,64	139,34	331,49	4,59	2,379	0,033
0	12,3	170,33	186,97	291,72	4,49	1,56	0,024
0	3	27,3	129,92	215,49	4,89	1,659	0,038
0	12,9	218,52	86,8	308,3	5,69	3,552	0,066
0	9,9	210,33	106,48	274,61	5,49	2,579	0,052
0	7,8	146,7	126,33	348,86	4,79	2,761	0,038
0	8,4	145,79	186,39	518,43	7,59	2,781	0,041
0	11,1	129,12	83,63	283,17	4,1	3,386	0,049
0	8,1	105,79	74,53	182,2	3,79	2,445	0,051
0	6,6	139,68	82,46	204,5	2,4	2,48	0,029
0	12	271,15	106,81	189,16	4,7	1,771	0,044
0	7,5	97,03	188,97	185,3	2,6	0,981	0,014
0	9,3	132,91	229,11	178,18	3,29	0,778	0,014
0	4,2	164,38	125,62	332	6,19	2,643	0,049
0	4,2	144,97	74,2	215,12	5,89	2,899	0,079
0	5,7	192,62	267,61	147,35	3	0,551	0,011
0	0,9	207,32	82,71	191,42	4,59	2,314	0,055
0	1,8	204,97	86,86	153,19	3,7	1,764	0,043
0	1,8	243,5	192,11	283,18	4,89	1,474	0,025
0	7,5	178,5			3,89		
0	9,6	132,62	91,45	149,82	3,2	1,638	0,035

0	13,8	156,15	61,76	174,86	4,39	2,831	0,071
0	5,4	156,15	51,23	196,12	4,49	3,828	0,088
0	6	125,56	76,66	298,7	6,49	3,896	0,085
0	2,7	149,09	146,68	229,7	5,09	1,566	0,035
0	10,8	154,97	135,25	193,68	5,49	1,432	0,041
0	7,0829	132,62	248,06	254,51	4,99	1,026	0,02
0	10,7555	116,74	325,37	330,66	6,69	1,016	0,021
0	3,9349	104,68	285,96	170,3	3,89	0,596	0,014
0	8,1322	228,79	117,5	225,73	5,79	1,921	0,049
0	9,4438	164,09	443,6	439,86	7,29	0,992	0,016
0	6,8205	239,38	178,87	402,71	7,59	2,251	0,042
0	14,1658	135,26	153	141,54	5,69	0,925	0,037
0	4,4596	118,21	423,44	207,07	6,69	0,489	0,016
0	2,6233	264,38	463,15	132,19	4,49	0,285	0,01
0	3,6726	101,44	618,68	337,42	7,29	0,545	0,012
0	9,1815	332,32	142,77	316,27	6,19	2,215	0,043
0	12,8541	121,74	202,94	182,36	4,59	0,899	0,023
0	11,5425	106,44	175,56	178,02	5,29	1,014	0,03
0	7,3452	104,97	106,37	328,1	6,39	3,085	0,06
0	7,8699	132,62	150,89	166,51	3,99	1,104	0,026
0	2,8856	117,91	237,83	229,36	4,39	0,964	0,018
0	2,0986	158,79	473,38	181,52	5,49	0,383	0,012
0	18,8877	162,91	797,06	191,17	5,69	0,24	0,007
0	9,7062	184,97	117,44	257,68	4,39	2,194	0,037
0	14,6904	96,15	278,45	286,34	6,29	1,028	0,023
0	9,1815	137,32	187,73	312,19	6,39	1,663	0,034
0	9,9685	234,09	142,93	466,66	8,09	3,265	0,057
0	4,9842	102,03	155,73	352,62	5,89	2,264	0,038
0	7,6075	226,74	306,33	179,47	4,89	0,586	0,016
0	7,0829	135,26	130,59	220,27	6,39	1,687	0,049
0	5,7712	277,91	265,74	261,8	6,69	0,985	0,025
0	11,0178	135,56	119,42	256,43	4,89	2,147	0,041
0	10,4932	164,97	128,97	136,76	3,5	1,06	0,027
0	9,9685	135,56	119,89	363,52	5,89	3,032	0,049
0	7,8699	86,74	184,59	352,82	6,39	1,911	0,035
0	8,1322	121,44	256,26	284,1	5,69	1,109	0,022
0	9,9685	133,79	166,21	421,93	6,99	2,539	0,042
0	10,4932	115,85	284,68	219,13	6,09	0,77	0,021
0	10,4495	100,26	104,18	203,2	4,89	1,95	0,047
0	8,2376	141,44	84,39	233,29	4,99	2,764	0,059
0	10,3732	92,32	89,17	135,52	5,89	1,52	0,066
0	10,4495	170,56	105,57	421,5	8,69	3,993	0,082
0	8,8477	161,44	291,98	148,22	4,89	0,508	0,017
0	10,6783	150,85	77,18	343,28	7,19	4,448	0,093
0	8,7715	221,44	116,98	382,61	10,09	3,271	0,086
0	7,9325	164,09	108,13	350,93	10,59	3,245	0,098
0	11,0597	67,32	108,37	351,04	6,49	3,239	0,06
0	9,3817	111,87	110,93	327,15	6,59	2,949	0,059
0	20,8227	200,55	105,23	342,02	8,99	3,25	0,085
0	13,2716	109,24	156,9	162,94	5,49	1,038	0,035
0	9,9156	127,39	99,87	172,53	8,19	1,728	0,082
0	8,924	121,08	85,09	351,96	5,99	4,136	0,07
0	9,8393	173,45	171,09	338,46	6,49	1,978	0,038
0	24,255	135,29	131,76	231,1	6,99	1,754	0,053
0	9,2291	140,55	42,85	468,75	11,88	10,939	0,277
0	8,6952	117,39	127,1	184,06	6,39	1,448	0,05
0	7,4748	98,97			8,89		
0	9,0003	99,24	349,34	487,37	8,99	1,395	0,026
0	7,5511	246,87	80,33	234,77	6,49	2,923	0,081
0	9,5342	190,29	109,12	341,66	8,09	3,131	0,074
0	9,5342	171,61	132,19	214,54	6,59	1,623	0,05
0	9,0766	92,39	270,44	209,75	6,39	0,776	0,024
0	11,0597	109,5	113,33	219,99	5,89	1,941	0,052
0	11,975	109,24	136,14	303,3	8,89	2,228	0,065
0	12,2038	116,34	160,47	413,29	8,29	2,575	0,052
0	8,3901	91,08	83,77	297,95	10,49	3,557	0,125
0	11,8224	107,66	116,13	243,62	9,69	2,098	0,083
0	9,9156	86,87	102,24	178,57	5,99	1,747	0,059

0	8,3901	109,5	321,94	186,21	6,69	0,578	0,021
0	9,3054	116,61	97,15	174,19	5,79	1,793	0,06
0	9,763	86,08	77,78	423,15	8,59	5,44	0,11
0	11,7066	108,45	104,54	170,88	4,59	1,635	0,044
0	6,8918	81,87	113,46	434,76	7,09	3,832	0,062
0	5,098	99,5	147,6	273,98	3,2	1,856	0,022
0	8,8743	120,82	255,47	361,13	7,99	1,414	0,031
0	8,6855	93,45	80,2	195,61	4,1	2,439	0,051
0	7,2694	186,87	94,98	276,55	5,89	2,912	0,062
0	7,2694	101,87	101,48	176,62	4,39	1,74	0,043
0	4,3428	146,08	96,51	330,73	1,9	3,427	0,02
0	10,8569	102,92	144,55	225,08	4,29	1,557	0,03
0	6,6086	190,55	147,99	211,48	9,29	1,429	0,063
0	7,2694	76,34	98,17	223,04	3,89	2,272	0,04
0	9,0632	185,29	96,51	518,37	8,49	5,371	0,088
0	10,9513	161,34	65,93	238,28	6,49	3,614	0,098
0	7,0806	126,87	150,1	408,56	6,89	2,722	0,046
0	7,7414	136,08	89,5	454,85	5,69	5,082	0,064
0	8,6855	87,92	111,19	230,23	3,89	2,071	0,035
0	13,0283	126,34	147,79	183,92	4,39	1,244	0,03
0	8,5911	206,87	53,03	720,2	7,29	13,581	0,137
0	7,9303	102,66	136,54	354,6	4,99	2,597	0,037
0	11,8954	138,71	140,47	510,58	9,39	3,635	0,067
0	10,9513	130,82	72,42	383,02	5,09	5,289	0,07
0	5,6645	199,5	93,84	563,83	8,19	6,008	0,087
0	8,5911	67,92	80,55	227,54	5,19	2,825	0,064
0	6,9862	115,29	122,58	500,65	7,29	4,084	0,059
0	6,8918	91,34	122,71	248,35	6,89	2,024	0,056
0	10,1016	87,66	98,18	383,31	3,1	3,904	0,032
0	7,7414	125,03	125,83	507,1	7,59	4,03	0,06
0	10,7625	117,13	73,37	492,56	6,39	6,713	0,087
0	9,0632	137,13	111,87	278,88	5,89	2,493	0,053
0	5,4757	66,34	115,26	369,19	5,69	3,203	0,049
0	6,1365	153,97	89,77	272,14	6,69	3,032	0,075
0	6,9862	85,82	142,91	459,82	7,09	3,218	0,05
0	9,724	75,03	109,29	346,12	4,59	3,167	0,042
0	0,4894	183,45	293,32	372,87	6,19	1,271	0,021
0	5,5062	121,19	101,57	293,1		2,886	0
0	6,3627	143,35	78,93	294,93		3,737	0
0	11,1347	127,68	183,58	378,24	4,59	2,06	0,025
0	10,2782	107,41	87,33	519,96	6,99	5,954	0,08
0	7,3416	136,59	96,41	273,95	5,59	2,842	0,058
0	12,4807	381,73	113,36	276,34	5,09	2,438	0,045
0	6,4851	134,43	117,02	375,01	3,89	3,205	0,033
0	2,4472	203,62	61,71	417,27	5,19	6,762	0,084
0	3,059	223,35	77,71	946,24	8,09	12,177	0,104
0	1,8354	119,3	121,22	259,51	3,5	2,141	0,029
0	8,4428	180,38	42,65	372,22	5,79	8,727	0,136
0	3,1813	273,89	76,18	320,54	6,49	4,208	0,085
0	7,3416	89,84	58,7	399,85	5,69	6,812	0,097
0	5,5062	96,59	81,86	654,91	7,29	8	0,089
0	3,6708	121,46	103,68	621,47	7,09	5,994	0,068
0	0,8	194,16	42,47	242,73	3,79	5,715	0,089
0	1,4683	151,46	58,44	358,4	5,49	6,133	0,094
0	0,8565	135,51	67,49	626,49	6,29	9,283	0,093
0	3,6708	134,97	100,39	261,92	5,29	2,609	0,053
0	4,8944	104,43	46,2	238,03	4,49	5,152	0,097
0	9,7888	113,89	102,88	567,62	6,29	5,517	0,061
0	7,4639	277,41	94,54	226,18	4,7	2,392	0,05
0	5,7509	114,43	92,77	235,07	4,79	2,534	0,052
0	7,4639	160,92	87,98	416,47	5,89	4,734	0,067
0	7,5863	145,78	81,77	235,6	4,59	2,881	0,056
0	12,1136	132	50,19	256,17	4,49	5,104	0,089
0	12,7254	152,81	85,67	334,13	4,99	3,9	0,058
0	10,4006	100,11	66,78	264,39	5,09	3,959	0,076
0	11,7465	177,14	60,48	284,19	5,69	4,699	0,094
0	11,3794	90,92	33,87	286,22	5,39	8,451	0,159
0	5,9956	216,59	65,09	372,86	5,29	5,728	0,081

0	4,2826	146,32	93,56	253,08	4,2	2,705	0,045
0	3,9155	181,46	60,3	412,48	6,49	6,84	0,108
0	1,8354	130,92	43,89	306,28	7,29	6,978	0,166
0	5,5062	95,24	36,97	386,75	5,09	10,461	0,138
0	9,9111	85,51	69,68	247,06	3,39	3,546	0,049
0	7,4639	81,19	36,61	191,82	2,7	5,24	0,074
0	11,3794	155,24	56,48	209,48	3,2	3,709	0,057
0	1,5907	143,35	79,25	585,61	4,79	7,389	0,06
0	3,4261	121,19	81,57	348,83	3,6	4,276	0,044
0	2,8143	165,51	45,89	723,52		15,766	0
0	7,0968	143,62	81,57	246,83	4,49	3,026	0,055
0	6,8521	142,81	62,72	203,61	3,7	3,246	0,059
0	6,4851	112	93,47	239,63	3,29	2,564	0,035
0	12,3583	132,81	119,57	388,81	3,39	3,252	0,028
0	7,9534	105,24	93,18	328,08	4,2	3,521	0,045
0	7,0968	12,27	102,6	217,65	2,79	2,121	0,027
0	9,6664	135,78	110,87	190,83	3,29	1,721	0,03
0	5,8733	114,16	99,27	265,45	5,69	2,674	0,057
0	30,1004	159,57	338,84	264,2	3,1	0,78	0,009
0	17,6198	191,19	85,2	229,31	3,2	2,691	0,038
0	26,0626	193,08	97,38	544,7	5,99	5,594	0,062
0	16,8856	143,89	111,02	223,14	3,39	2,01	0,031
0	7,4639	116,39	77,51	390,37	4,7	5,036	0,061
0	7,831	141,13	205,14	235,52	3,7	1,148	0,018
0	4,5273	145,87	120,73	490,67	7,59	4,064	0,063
0	8,8099	85,61	111,31	324,99	5,69	2,92	0,051
0	15,1726	142,18	115,37	218,24	4,2	1,892	0,036
0	8,1981	153,24	200,56	254,09	8,89	1,267	0,044
0	7,3416	234,55	140,31	264,95	9,99	1,888	0,071
0	11,8689	160,87	145,97	483,48	8,39	3,312	0,057
0	44,1718	157,71	92,89	654,15	6,19	7,042	0,067
0	6,6074	91,92	160,04	576,5	6,99	3,602	0,044
0	15,0502	190,34	137,99	666,3	7,79	4,829	0,056
0	8,4428	139,82	121,17	296,76	4,99	2,449	0,041
0	7,831	220,61	95,17	263,03	5,89	2,764	0,062
0	5,6471	146,66	81,98	343,52	5,59	4,19	0,068
0	16,5882	157,18	94,51	461,43	5,79	4,882	0,061
0	8,1176	112,18	94,79	349,1	4,1	3,683	0,043
0	11,6471	101,66	79,74	335,43	6,49	4,207	0,081
0	15,1765	143,24	91,71	415,69	5,59	4,533	0,061
0	12,7059	290,87	120,88	508,1	6,19	4,203	0,051
0	13,4118	331,39	46,64	238,94	2,5	5,123	0,054
0	13,0588	131,66	171	370,42	4,7	2,166	0,027
0	7,7647	184,82	63,66	214,92	4,1	3,376	0,064
0	4,2353	142,71	58,23	516,12	7,29	8,863	0,125
0	12,7059	141,13	67,21	364,26	4,59	5,42	0,068
0	11,2941	89,82	103,67	340,33	3,3	3,283	0,032
0	14,8235	286,13	66,37	201,7	3,4	3,039	0,051
0	9,5294	156,66	70,39	234,11	4,29	3,326	0,061
0	17,2941	220,87	57,3	509,22	6,19	8,887	0,108
0	7,0588	129,03	66,55	527,88	6,19	7,932	0,093
0	3,5294	187,45	38,13	219,77	3	5,764	0,079
0	6	307,71	78,71		3,5		0,044
0	6	97,45	45,61		5,49		0,12
0	12	151,66	63,94		4,89		0,076
0	12,7059	90,08	83,29	435,1	5,09	5,224	0,061
0	8,8235	84,29	64,12	177,46	1,5	2,768	0,023
0	10,2353	119,82	61,88	286,42	5,09	4,629	0,082
0	7,7647	144,55	58,79	291,08	8,19	4,951	0,139
0	14,8235	114,03	51,97	531,93	5,99	10,235	0,115
0	8,8235	93,24	105,91	644,21	5,59	6,083	0,053
0	12	228,5	51,93	515,05	5,09	9,918	0,098
0	12,7059	147,18	93,59	239,65	3,2	2,561	0,034
0	16,9412	166,39	71,28	278,73	5,29	3,91	0,074
0	14,1176	142,45	44,53	493,83	6,99	11,09	0,157
0	5,2941	134,55	100,81	607,76	6,09	6,029	0,06
0	4,9412	9,29	77,12	266,13	4,59	3,451	0,06
0	4,2353	134,29	43,51	315,26	6,79	7,246	0,156

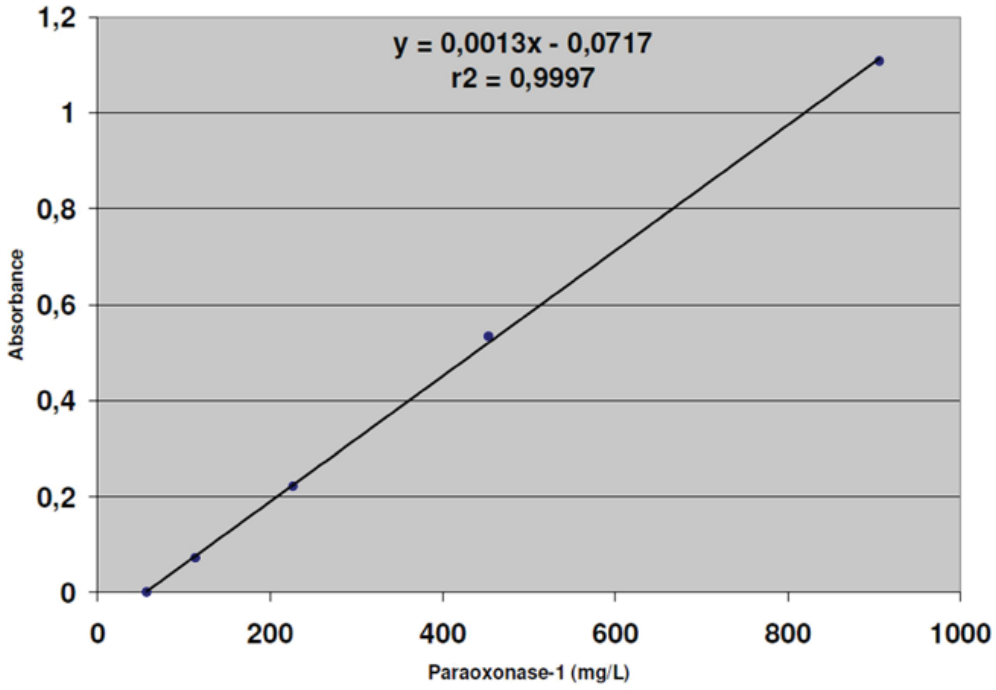
0	9,1765	119,03	64,34	237,33	4,2	3,689	0,065
0	4,5882	164,82	67,95	392,59	4,79	5,778	0,07
0	6,7059	306,13	26,85	238,94	4,99	8,899	0,186
0	49,4118	299,29	23,79	315,42	5,49	13,259	0,231
0	8,1176	114,82	37,77	240,08	4,39	6,356	0,116
0	13,7647	195,87	44,99	480	7,49	10,669	0,166
0	20,1176	12,97	76,37	349,91	4,29	4,582	0,056
0	4,9412	89,55	59,8	320,09	5,79	5,353	0,097
0	6	171,66	61,66	265,71	4,89	4,309	0,079
0	11,6471	115,39	53,32	191,51	3,29	3,592	0,062
0	7,4118	158,03	36,85	594,11	6,29	16,122	0,171
0	12,3529	163,82	59,34	341,39	3,89	5,753	0,066
0	11,2941	147,24	50,27	360,92	5,99	7,18	0,119
0	10,5882	115,66	79,34	262,13	4,7	3,304	0,059
0	6,3529	112,76	63,97	438,41	4,89	6,853	0,076
0	5,6471	73,82	79,71	615,82	6,49	7,726	0,081
0	8,4706	99,61	51,38	431,05	5,59	8,389	0,109
0	31,7647	133,29	68,88	322,13	5,09	4,677	0,074
0	12,7059	84,34	52,68	255,05	4,1	4,841	0,078
0	10,9412	129,61	81,93	478,64	5,49	5,842	0,067
0	5,2941	207,5	114,98	255,77	4,29	2,224	0,037
0	6,3529	210,92	61,53	282,9	4,2	4,598	0,068
0	5,2941	235,13	61,98	487,73	5,79	7,869	0,093
0	4,9412	131,45	31,49	483,76	5,79	15,362	0,184
0	7,4118	151,71	23,5	214,06	4,2	9,109	0,179
0	7,7647	206,45	61,53	227,26	4,1	3,693	0,067
0	7,4118	100,13	25,17	538,88	6,19	21,41	0,246
0	10,9412	186,45	96,24	315,93	5,09	3,283	0,053
0	12,3529	134,08	144,13	284,31	6,29	1,973	0,044
0	14,4706	140,66	91,47	333,77	5,29	3,649	0,058
1	84,09	484	43,47	157,05	1,368	3,613	0,031
1	140,91	544	43,338	86,26	1,208	1,99	0,028
1	97,73	1403	68,028	90,85	0,775	1,335	0,011
1	90,91	428	40,843	128,34	1,505	3,142	0,037
1	70,45	509	22,851	182,11	2,44	7,969	0,107
1	65,91	1144	20,619	94,09	1,414	4,563	0,069
1	65,91	442	25,872	518,23	4,811	20,031	0,186
1	102,27	813	40,581	101,19	1,026	2,494	0,025
1	68,18	539	37,56	231,29	1,778	6,158	0,047
1	72,73	955	27,185	266,33	4,378	9,797	0,161
1	63,64	936	22,983	272,23	3,124	11,845	0,136
1	54,55	753	22,195	146,64	2,713	6,607	0,122
1	72,73	415	29,943	221,28	1,3	7,39	0,043
1	122,73	844	13,79	154,01	2,348	11,168	0,17
1	65,91	740	23,508	120,33	2,12	5,119	0,09
1	113,64	696	27,316	223,39	3,352	8,178	0,123
1	93,18	637	25,215	136,9	3,032	5,429	0,12
1	120,45	940	14,315	137,62	2,098	9,614	0,147
1	79,55	479	25,215	116,66	2,052	4,627	0,081
1	68,18	663	17,992	300,98	3,352	16,729	0,186
1	118,18	534	35,196	142,28	1,573	4,043	0,045
1	109,09	544	18,386	137,29	2,736	7,467	0,149
1	111,36	895	24,033	79,45	0,638	3,306	0,027
1	115,91	1154	30,731	222,27	2,759	7,233	0,09
1	68,18	750	12,87	187,05	2,987	14,534	0,232
1	86,36	643	26,66	146,49	1,87	5,495	0,07
1	45,45	753	14,446	375,82	3,306	26,016	0,229
1	115,91	568	22,063	83,5	0,547	3,785	0,025
1	213,64	1032	26,266	544,63	5,176	20,735	0,197
1	120,45	587	19,962	172,71	4,241	8,652	0,212
1	102,27	1048	15,759	236,29	3,055	14,994	0,194
1	172,73	579	42,813	220,93	2,417	5,16	0,056
1	120,45	660	13,396	134,9	3,534	10,07	0,264
1	172,73	404	16,285	155,95	3,352	9,576	0,206
1	84,09	371	30,468	174,16	3,055	5,716	0,1
1	109,09	1015	24,427	177,04	2,166	7,248	0,089
1	88,64	678	16,679	80,97	1,436	4,855	0,086
1	54,55	423	15,103	281,96	3,602	18,669	0,238

1	70,45	721	26,528	181,63	3,466	6,847	0,131
1	43,18	516	12,54	198,09	4,355	15,797	0,347
1	134,09	1172	28,869	179,03	2,827	6,201	0,098
1	54,55	453	31,025	315,7	3,944	10,176	0,127
1	113,64	451	25,942	194,08	4,286	7,481	0,165
1	59,09	830	23,785	218,21	4,058	9,174	0,171
1	111,36	2450	59,062	281,23	3,671	4,762	0,062
1	106,82	401	18,393	295,55	3,899	16,069	0,212
1	70,45	609	32,72	168,56	4,172	5,152	0,128
1	106,82	983	14,388	239,16	2,166	16,622	0,151
1	54,55	524	25,017	355,9	5,609	14,226	0,224
1	118,18	1057	23,015	99,32	2,394	4,315	0,104
1	131,82	1118	54,749	171,48	5,951	3,132	0,109
1	86,36	721	38,574	169,71	3,192	4,4	0,083
1	100	1224	42,579	106,25	2,554	2,495	0,06
1	184,09	876	26,866	264,62	3,26	9,85	0,121
1	86,36	531	16,545	156,53	2,736	9,461	0,165
1	93,18	211	17,777	89,62	3,078	5,041	0,173
1	113,64	243	32,72	227,43	5,404	6,951	0,165
1	77,27	384	19,318	250	3,055	12,941	0,158
1	84,09	524	19,934	111,55	3,488	5,596	0,175
1	59,09	910	21,782	124,09	2,599	5,697	0,119
1	115,91	539	42,733	215,98	3,329	5,054	0,078
1	79,55	529	53,054	296,83	4,993	5,595	0,094
1	163,64	359	58,292	118,58	6,635	2,034	0,114
1	113,64	293	9,613	239,87	5,404	24,953	0,562
1	81,82	342	41,963	77,41	2,28	1,845	0,054
1	131,82	245	69,999	97,8	1,984	1,397	0,028
1	106,82	740	29,485	113,02	2,166	3,833	0,073
1	70,45	326	52,13	194,24	3,078	3,726	0,059
1	68,18	1550	33,182	83,48	2,234	2,516	0,067
1	100	350	27,02	218,13	6,156	8,073	0,228
1	86,36	357	29,947	284,19	6,316	9,49	0,211
1	81,82	598	36,109	132,64	4,264	3,673	0,118
1	93,18	489	27,328	198,06	3,625	7,248	0,133
1	122,73	499	47,816	336,88	6,316	7,045	0,132
1	65,91		25,788	193,14	2,918	7,49	0,113
1	131,82	702	51,051	171,02	3,83	3,35	0,075
1	45,45	491	55,25	177,57	6,27	3,214	0,113
1	168,18	1389	35,112	110,6	2,804	3,15	0,08
1	170,45	359	77,499	217,49	2,736	2,806	0,035
1	45,45	950	45,018	137,79	3,146	3,061	0,07
1	65,91	521	24,231	210,97	4,172	8,707	0,172
1	95,45	500	39,172	240,83	3,648	6,148	0,093
1	45,45	272	20,333	289,75	5,449	14,25	0,268
1	68,18	488	50,702	234,15	5,54	4,618	0,109
1	29,55	405	24,393	151,15	5,426	6,196	0,222
1	43,18	673	22,931	89,44	2,736	3,9	0,119
1	65,91	849	28,453	136,91	5,312	4,812	0,187
1	43,18	582	14,811	175,34	6,931	11,838	0,468
1	106,82	636	43,719	141,21	3,26	3,23	0,075
1	77,27	368	24,393	615,84	7,752	25,247	0,318
1	90,91	345	49,728	195,83	5,7	3,938	0,115
1		1142	65,968	83,2	1,642	1,261	0,025
1		775	14,811	181,02	2,964	12,222	0,2
1	154,55	605	12,538	84,01	0,752	6,7	0,06
1	109,09	542	16,435	293,22	2,417	17,841	0,147
1	45,45	764	19,846	152,34	2,554	7,676	0,129
1	145,45	413	23,094	127,55	3,169	5,523	0,137
1	65,91	289	29,915	247,65	3,944	8,278	0,132
1	88,64	787	21,307	397,42	4,15	18,652	0,195
1	63,64	438	62,396	262,71	2,417	4,21	0,039
1	29,55	643	36,086	119,25	3,466	3,305	0,096
1	120,45	784	42,095	159,52	2,303	3,79	0,055
1	38,64	586	28,128	324,04	3,306	11,52	0,118
1	84,09	720	19,846	116,85	3,762	5,888	0,19
1	22,73	704	62,558	232,65	2,508	3,719	0,04
1		753	57,036	360,34	3,101	6,318	0,054

1	145,45	413	40,633	100,79	2,782	2,48	0,068
1	77,27	742	31,864	176,8	1,505	5,549	0,047
1	127,27	638	76,037	85,76	1,414	1,128	0,019
1	188,64	673	24,555	84,12	1,596	3,426	0,065
1	90,91	292	35,112	163,85	3,124	4,666	0,089
1	122,73	144	56,874	328,17	3,146	5,77	0,055
1	81,82	285,6	12,4	160,3	3,12	12,92	0,25
1	70,45	623,4	50,3	168,63	1,19	3,35	0,023
1	86,36	1015,3	70,2	151,84	2,74	2,16	0,039
1	95,45	723,4	37,3	301,27	2,65	8,07	0,071
1	140,91	410,1	15,3	487,63	6,56	31,87	0,428
1	90,91	357,2	26,4	267,69	3,18	10,13	0,012
1	81,82	812,4	28,3	250,1	4,1	8,83	0,144

SUPPLEMENTARY FIGURE 1: Representative example of a calibration curve of our paraoxonase-1 in house ELISA

Note: This curve has been drawn with the Spanish version of Excel® program, in which decimal numbers are separated from the integer by a comma, not by a decimal point.



SUPPLEMENTARY TABLE 4: Plasma F_2-isoprostanes concentrations in patients with peripheral artery disease segregated according to whether they took or not a particular medication.

Differences assessed by the Mann-Whitney *U* test.

Medication	Yes	No	*p*
Antiplatelet drugs	86.4 (45.5 – 163.6)	95.5 (44.3 – 175.0)	0.499
Statins	90.9 (44.6 – 186.7)	91.9 (44.2 – 157.9)	0.814
Antidiabetic drugs	95.5 (61.8 – 271.4)	90.9 (65.7 – 178.9)	0.691
Angiotensin converting enzyme inhibitors	95.5 (61.8 – 271.4)	90.9 (65.7 – 178.9)	0.691
Angiotensin receptor antagonists	88.6 (59.7 – 153.8)	85.2 (54.6 – 259.9)	0.384
Calcium receptor antagonists	88.6 (61.6 – 172.3)	90.9 (68.2 179.1)	0.426
Diuretics	88.6 (43.6 – 169.1)	90.9 (45.1 – 185.7)	0.975
Anti-arrhythmia drugs	90.9 (63.2 – 170.2)	177.3 (54.5 – 306.8)	0.387
Beta-blockers	90.9 (44.1 – 172.7)	93.2 (50.0 – 163.6)	0.123
Bronchodilators	89.8 (66.6 – 230.0)	106.8 (59.1 – 177.3)	0.523

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ESTUDIO 2

Galectin-3 in peripheral artery disease. Relationships with markers of oxidative stress and inflammation

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Abstract: Galectin-3 is a modulator of oxidative stress, inflammation and fibrogenesis involved in the pathogenesis of vascular diseases. The present study sought to characterize, in patients with peripheral artery disease (PAD), the localization of galectin-3 in arterial tissue, to analyze the relationships between the circulating levels of galectin-3 and oxidative stress and inflammation, and to compare the diagnostic accuracy of galectin-3 with that of other biochemical markers of this disease. We analyzed femoral or popliteal arteries from 50 PAD patients, and 4 control arteries. Plasma from 86 patients was compared with that from 72 control subjects. We observed differences in the expression of galectin-3 in normal arteries and arteries from patients with PAD, with a displacement of the expression from the *adventitia* to the *media* and the *intima*. In addition, plasma galectin-3 concentration was increased in PAD patients and correlated with serologic markers of oxidative stress (F2-isoprostanes) and inflammation [chemokine (C-C motif) ligand 2, C-reactive protein, β-2-microglobulin]. We conclude that the determination of galectin-3 has good diagnostic accuracy in the assessment of PAD, and compares well with other analytical parameters currently in use.

1. Introduction

Galectins are a lectin family able to bind to β-galactoside groups [1]. To-date, 14 mammalian galectins have been identified, all of which contain a carbohydrate-recognition-binding domain of 130 amino acids. Galectin-3 is a 29- to 35-kDa protein consisting of two domains, the C-terminal carbohydrate-recognition-binding-domain, and the N-terminal domain that has a unique short end continuing into a pro-gly-ala-tyr-rich

repeat motif [2]. Galectin-3 has been recognized increasingly as a modulator of multiple biological functions such as oxidative stress, proliferation, macrophage chemotaxis, phagocytosis, neutrophil extravasation, neutrophil migration during venous thrombosis, apoptosis, vacuole lysis after infection, fibrogenesis and angiogenesis [3-6]. Relationships with oxidative stress have been demonstrated *in vitro* such that treatment of monocytes with phorbol myristate acetate, a NADPH oxidase-dependent inducer of reactive oxygen species, produced an increase in galectin-3 mRNA and protein expression, while blockade with apocynin reversed these effects [7].

Recent evidence suggests that galectin-3 plays a role in the pathogenesis of numerous disease conditions including cancer as well as inflammatory and metabolic disorders [8-11]. The role of galectin-3 in atherosclerosis deserves special attention. Inactivation of galectin-3 gene or therapeutic modulation of the protein levels was shown to halt the progression of cardiac remodeling, attenuate myocardial fibrogenesis, reduce the atherosclerotic lesion size, and preserve ventricular function in rats and mice [12-15]. Also, galectin-3 is involved in vascular smooth muscle cell osteogenic differentiation [16], myocardial fibrosis and inflammation [17] together with a strong suggestion of association with the risk of cardiac fibrosis, hearth failure, and mortality in the general population and in patients with atherosclerosis [7,18-20].

In contrast to cardiac disease, the information on galectin-3 in peripheral artery disease (PAD) is scarce. Lower-extremity PAD is a frequent manifestation of atherosclerosis that is associated with extensive impairment of different arterial territories. The prevalence of this disease increases with age and, in people over the age of 70 years, it is estimated to be about 20% [21]. Oxidative stress and inflammation are

important factors for the initiation and progression of PAD, and the inflammatory mediators involved in this process are similar to those contributing to coronary artery disease [22,23]. A differential characteristic of PAD is that the associated atherosclerosis affects much more extensive regions of arteries and, consequently, the associated biochemical alterations are often more marked than in coronary artery disease [24]. The possibility of finding efficient non-invasive biomarkers for the early diagnosis of PAD is currently being investigated, and several studies proposed C-reactive protein (CRP) measured by a high-sensitivity method, or β-2-microglobulin (B2M) as useful markers of this disease [25-26]. We recently reported that the measurement of the serum concentrations of F2-isoprostanes and/or the chemokine (C-C motif) ligand 2 (CCL2) which are markers of oxidative stress and inflammation, may also constitute excellent biomarkers for the diagnosis of PAD [27].

The present study characterized the localization of galectin-3 in arterial tissue, and evaluated the relationships between the circulating levels of galectin-3 *versus* oxidative stress and inflammation. The diagnostic accuracy of galectin-3 compared favorably with other biochemical markers of these processes, in patients with PAD relative to control individuals.

2. Results

2.1. Histological and immunohistochemical analyses

In normal arteries, galectin-3 expression was evenly distributed throughout the *tunica adventitia* of the artery wall. We observed increased staining in certain areas, coinciding with inflammatory infiltrates. We did not see any specific staining in the *tunica media* or the *tunica intima*. We observed a faint positive labeling for CD68 antigen (a

marker of macrophages) in the *adventitia*, but not in the *media* or the *intima*, and a positive labeling for α-actin (a marker of smooth muscle cells) in the *media* (Figure 1).

Arterial samples from patients with PAD had an increased *intima* thickness with respect to the *media* [*intima*/*media* ratio: 2.06 (0.52 – 6.19) *vs.* 0.13 (0.10 – 0.16); $p = 0.002$]. Subjects with the less thickened *intima* layer showed galectin-3 localization mostly together with smooth muscle cells of the *media*, and some positive staining in the remains of inflammatory infiltrates around the vessels of the *adventitia,* but not in the *intima* (Figure 2). In contrast, arteries from patients with the more thickened *intima* showed staining for galectin-3, CD68 and α-actin in the *intima* (Figure 3).

There were no any significant differences in the percentage positive staining for galectin-3 between patients and controls [3.55 (0.60 – 13.69)% *vs.* 7.11 (0.93 – 11.04)%, respectively; $p = 0.391$].

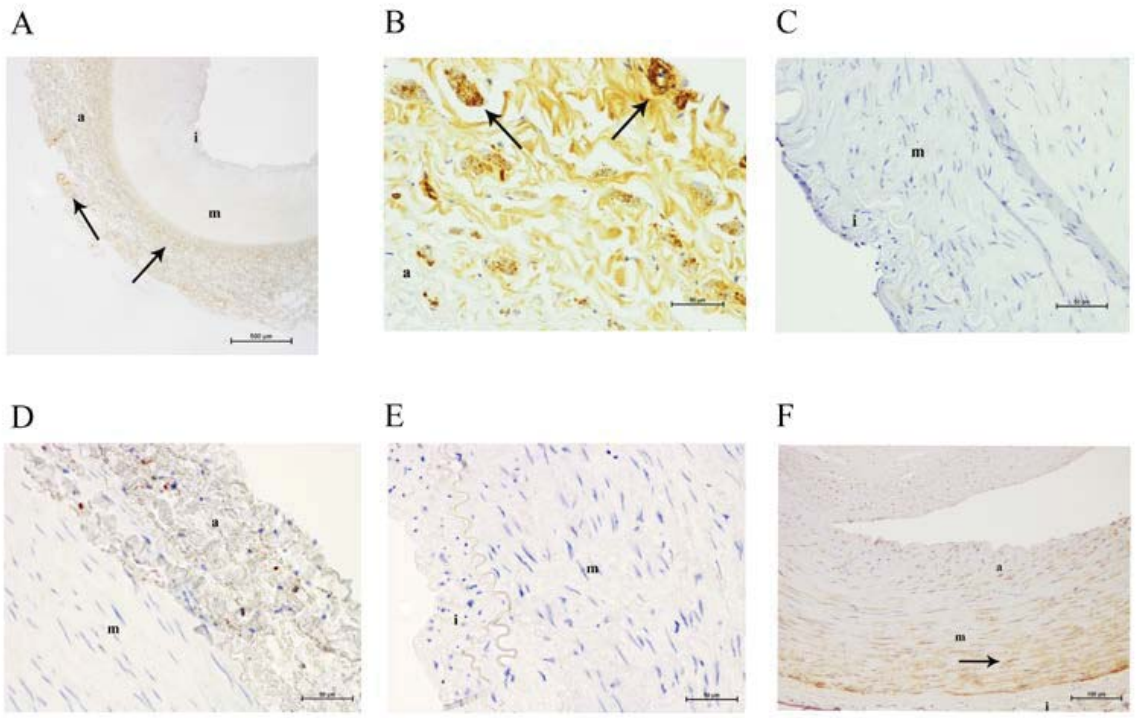


Figure 1. Immunohistochemical images of a normal femoral artery. **A)** Galectin-3 staining in *tunica adventitia* at 40X . **B)** Galectin-3 staining in the *adventitia* at 200X. **C)** Lack of galectin-3 staining in *tunica media* at 200X. **D)** Faint CD68 staining in the *adventitia* at 200X. **E)** Lack of CD68 staining in the *media* at 200X; **F)** α-actin staining in the media at 100X. **a**, *adventitia*; **m**, media; **i**, *intima*. The arrows show positive immunostained areas.

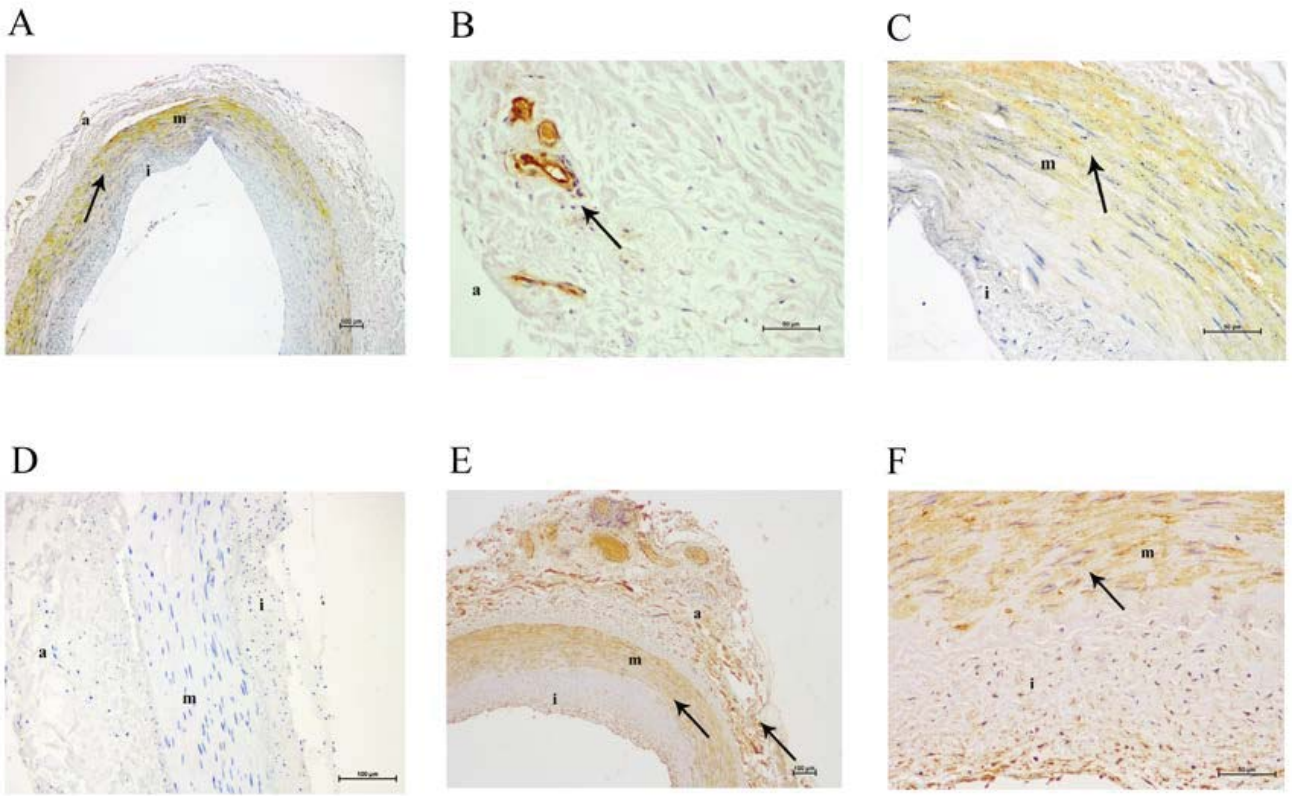


Figure 2. Immunohistochemical images of a moderately altered femoral artery. **A)** Galectin-3 staining at 40X . **B)** Galectin-3 staining in *tunica adventitia* at 200X. **C)** Galectin-3 staining in *tunica media* at 200X. **D)** Lack of CD68 staining at 100X. **E)** α-actin staining at 40X. **F)** α-actin staining at 200X. **a**, *adventitia*; **m**, media; **i**, *intima*. The arrows show positive immunostained areas.

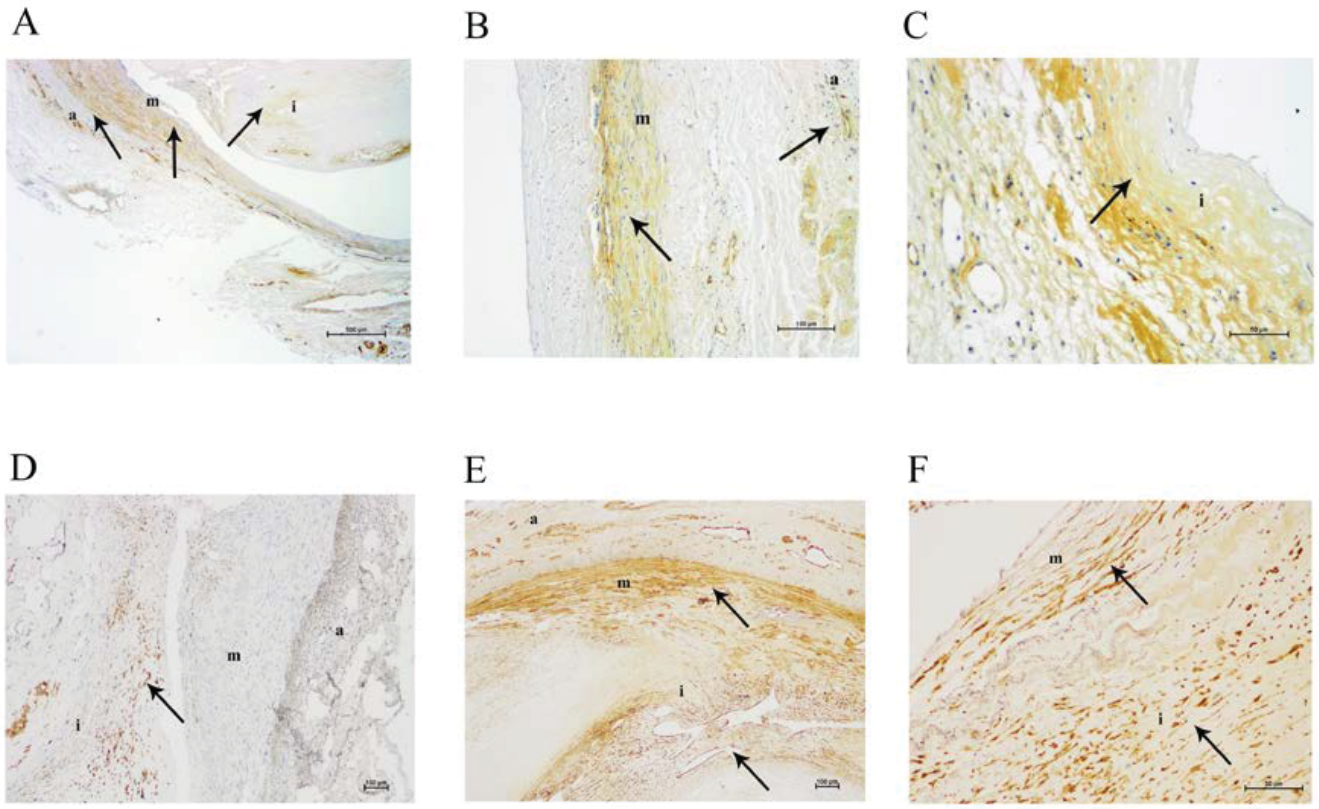


Figure 3. Immunohistochemical images of a severely altered femoral artery. **A)** Galectin-3 staining at 40X . **B)** Galectin-3 staining in *tunica adventitia* at 100X. **C)** Galectin-3 staining in t*unica intima* at 200X. **D)** CD68 staining at 100X **E)** α-actin staining in *adventitia*, *media*, and *intima* at 100X. **F)** α-actin staining in *media* and *intima* at 200X. **a**, *adventitia*; **m**, media; **i**, *intima*. The arrows show positive immunostained areas.

2.2. Biochemical analyses

Fifty-three percent of patients were at stages III and IV of the Fontaine classification, and 46% were at stages I and II. There were no significant differences between patients and controls with respect to age and smoking habit. PAD patients were more frequently male, and more had arterial hypertension, diabetes mellitus, dyslipidemia, ischemic heart disease and chronic obstructive pulmonary disease. There were significant increases in plasma galectin-3, F2-isoprostanes, CCL2, B2M, and CRP concentrations in PAD patients, compared with the control group (Table 1). Plasma galectin-3 and age were not significantly correlated (r = 0.142; p = 0.076). There were no significant differences in plasma galectin-3 concentrations when patients were segregated according to the other clinical and demographic variables (Table 2). We found significant direct correlations between plasma galectin-3 and F2-isoprostanes, CCL2, CRP, and B2M concentrations (Table 3).

We subsequently segregated PAD patients according to mild disease (Fontaine Stage I+II) or severe disease (Fontaine Stage III+IV); we did not observe any significant differences either in plasma galectin-3 concentrations or in any of the other biochemical variables studied; the concentrations were increased with respect to the control group (Fig. 4).

The diagnostic accuracy of all the selected variables in discriminating between the hualthy volunteers and the patients with PAD was high. However, plasma galectin-3 measurement was not more efficient than the classical CRP and B2M markers. The order of the calculated accuracies was: F2-isoprostanes ≅ CCL2 > CRP ≅ B2M ≅ galectin-3 (Fig. 5). The ratios between galectin-3 and B2M or CRP significantly improved the diagnostic accuracy of galectin-3 alone (Fig. 6).

Table 1. Demographic, clinical and biochemical characteristics of the control group and peripheral artery disease (PAD) patients.

Variable	Control group (n = 72)	PAD (n = 86)	*p*-Value
Age, years	63 (59 – 73)	66 (49 – 87)	0.529
Male gender, n (%)	47 (65.3)	68 (79.1)	0.039
Smoking, n (%)	17 (23.6)	6 (10.9)	0.048
Medications, n (%):			
Antiplatelet drugs		25 (29.0)	
Statins		48 (55.8)	
Antidiabetic drugs		48 (55.8)	
Angiotensin converting enzyme		48 (55.8)	
inhibitors		41 (47.6)	
Angiotensin receptor antagonists	NR	55 (63.9)	
Calcium receptor antagonists		46 (53.5)	
Diuretics		73 (84.9)	
Anti-arrhythmia drugs		73 (84.9)	
Beta-blockers		59 (68.6)	
Bronchodilators			
Arterial hypertension, n (%)	12 (16.9)	28 (63.6)	<0.001
Diabetes mellitus, n (%)	4 (5.6)	29 (67.4)	<0.001
Dyslipidemia, n (%)	7 (9.9)	20 (46.5)	<0.001
Ischemic heart disease, n (%)	0	4 (22.2)	
Chronic obstructive pulmonary disease, n (%)	0	9 (25.0)	
Ankle brachial index = 0.4 – 0.9	NA	81 (94.2)	
Ankle brachial index < 0.4	NA	5 (5.8)	
Fontaine classification			
Stage I, n (%)	NA	3 (3.4)	
Stage II, n (%)	NA	37 (43.0)	
Stage III, n (%)	NA	9 (10.5)	
Stage IV, n (%)	NA	37 (43.0)	
Galectin-3, ng/mL	6.13 (3.05 – 12.2)	10.79 (4.21 – 19.09)	<0.001
F2-isoprostanes, pg/mL	7.76 (3.03 – 14.82)	90.91 (47.62 – 141.71)	<0.001
Chemokine (C-C motif) ligand 2, pg/mL	136.34 (88.37 – 203.22)	565.75 (211.00 – 1154.00)	<0.001
β-2-microglobulin, mg/L	1.53 (1.09 – 2.35)	2.22 (1.34 – 4.55)	<0.001
C-reactive protein, mg/L	0.19 (0.02 – 0.74)	0.80 (0.07 – 2.82)	<0.001

Table 2. Plasma galectin-3 concentrations (ng/mL) in peripheral artery disease (PAD) patients segregated according to the presence or absence of the selected clinical variables.

Variable	No	Yes	*p*-Value
Male gender	11.22 (5.50 – 19.13)	10.60 (4.07 – 19.98)	0.361
Smoking	10.84 (4.57 – 19.00)	10.53 (18.89 – 26.55	0.948
Arterial hypertension	8.79 (3.29 – 16.76)	10.64 (5.13 – 23.12)	0.222
Diabetes mellitus	10.57 (3.29 – 24.69)	10.18 (4.08 – 19.00)	0.726
Dyslipidemia	10.10 (3.46 – 20.61)	10.20 (4.11 – 24.41)	0.932
Ischemic heart disease	8.79 (6.55 – 18.31)	9.43 (4.88 – 24.69)	0.878
Chronic obstructive pulmonary disease	9.72 (4.06 – 22.14)	11.45 (3.29 – 21.19)	0.349

Table 3. Correlations between plasma galectin-3 concentrations and the other selected biochemical variables.

Parameter	Spearman's ρ	*p*-Value
F2-isoprostanes	0.437	<0.001
Chemokine (C-C motif) ligand	0.295	0.005
C-reactive protein	0.341	<0.001
β-2-microglobulin	0.544	<0.001

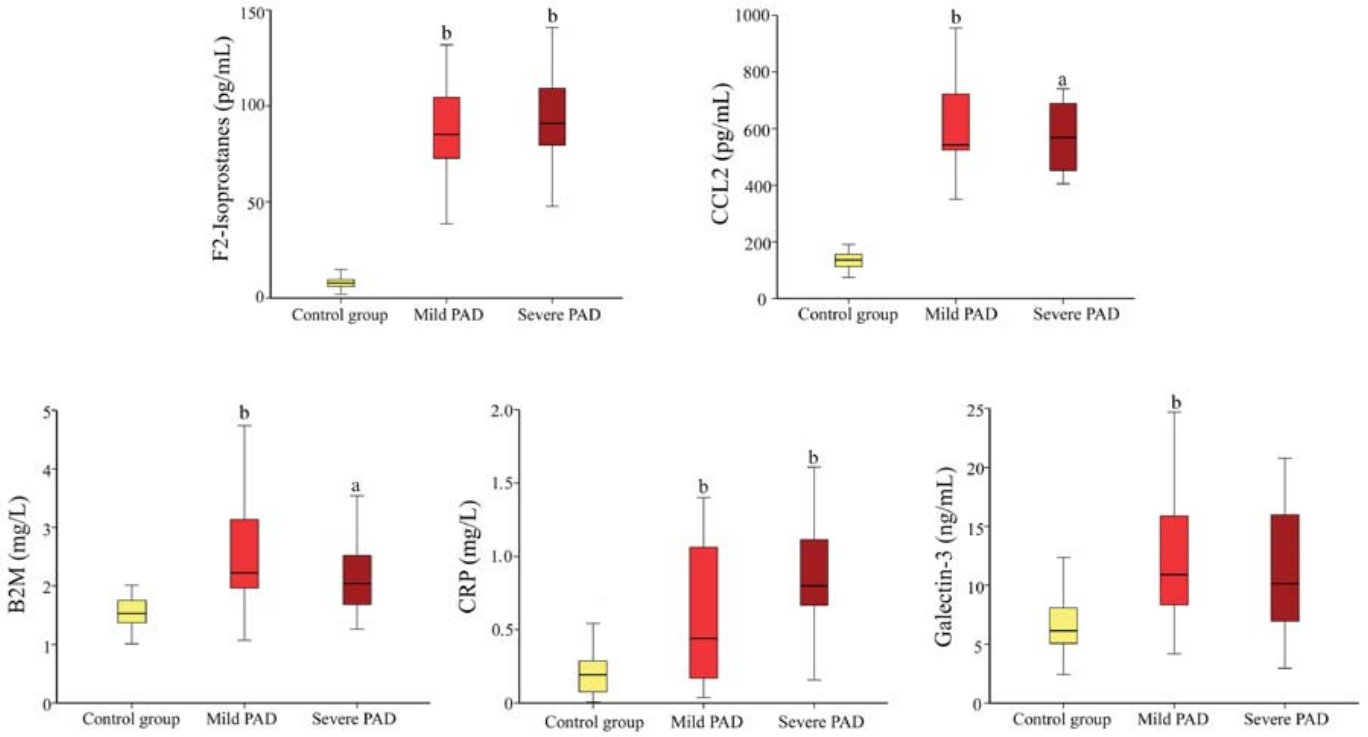
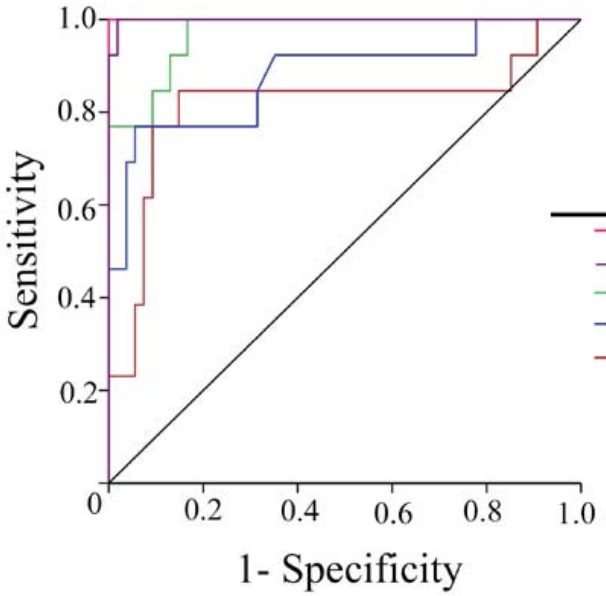
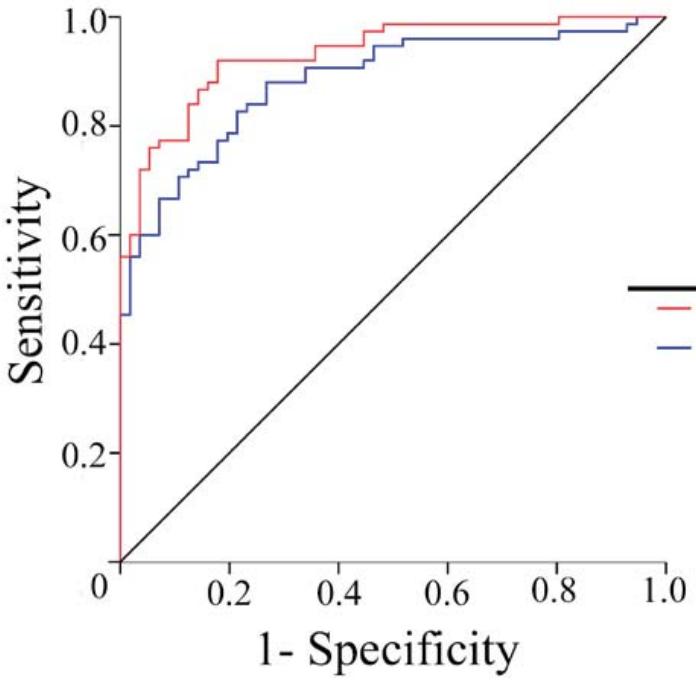


Figure 4. Selected biochemical variables in PAD patients classified according to whether they had mild disease (Fontaine Stages I and II) or severe disease (Fontaine Stages III and IV) and in the control group. Significance values by the Mann-Whitney *U* test: [a]$p<0.01$; [b]$p<0.001$, with respect to the control group.



		Variable	*AUROC*	*SE*	*95% CI*
—	1	F2-isoprostanes	1	<0.001	1.000-1.000
—	2	CCL2	0.999	0.002	0.994-1.000
—	3	CRP	0.97	0.019	0.932-1.000
—	4	B2M	0.877	0.064	0.753-1.000
—	5	Galectin-3	0.813	0.086	0.645-0.982

Figure 5. Receiver operating characteristics (ROC) plots for all the studied biomarkers in PAD patients and in the control group. AUROC: areas under the curve of the ROC plots. SE: Standard Error.



		Variable	AUROC	SE	95% CI
—	1	Galectin-3/CRP	0.932	0.021	0.891-0.973
—	2	Galectin-3/B2M	0.882	0.029	0.825-0.939

Figure 6. Receiver operating characteristics (ROC) plots for the galectin-3/CRP and galectin-3/B2M ratios in PAD patients and in the control group. AUROC: areas under the curve of the ROC plots. SE: Standard Error.

3. Discussion

Galectins, particularly galectin-3, have been gaining importance recently as significant components within the cascade of events underlying the inflammatory reaction and fibrogenesis caused by oxidative stress [2]. Distributions in normal and diseased human peripheral arterial tissue have yet to be defined. Galectin-3 is known to be expressed in a variety of organs in the mouse including the kidney, lung, spleen, colon, uterus, and ovary [28]. In apolipoprotein E-deficient mice (a model of experimental atherosclerosis) galectin-3 is expressed in macrophage-rich areas, but not in smooth muscle cell-rich areas of aortic roots and brachiocephalic arteries [13]. Results of the present study show important differences in galectin-3 distribution in normal arteries and arteries from PAD patients. Normal arteries showed galectin-3 expression exclusively in the *adventitia*, while arteries from patients showed the localization mainly adjacent to the smooth muscle cells of the *media*, with some minor staining in the *adventitia* and the *intima*. The pathophysiological interpretation of this finding cannot be inferred from the present investigation, but previous data suggest that this differential tissue distribution could be related to the processes of inflammation, fibrogenesis and calcification of the atherosclerotic plaque. Indeed, patients with an advanced PAD and atheroma plaque expressed galectin-3 together with macrophages in the *intima*, suggesting a coordinated role in the formation of the plaque. This hypothesis is supported by data demonstrating that galectin-3 stimulates myofibroblast activation and fibrosis in several types of cells [29,30]. In aortic valves from patients with aortic stenosis, galectin-3 co-localized with the α-smooth muscle cell markers actin and vimentin, and with

osteogenic markers such as osteopontin, bone morphogenetic protein 2, runt-related transcription factor 2, and sex-determining region Y-box 9 [31]. Reports indicate that human carotid plaques express galectin-3 in smooth muscle cells, especially in macrocalcified areas where it co-localizes with alkaline phosphatase [16]. Of note from an early study from our group is the observation of calcium deposition in the *media* of arteries from PAD patients; a distinct and frequent finding in affected arteries [32]. In the same study we reported an increase in CD68-positive cells in diseased arteries; the conclusion drawn is that, under certain stimuli, arterial smooth muscle cells can shift their phenotypes to a macrophage-cell state [33]. In addition, another study from our group showed a similar distribution of CCL2 and its receptors in the *intima* of PAD patients with advanced disease and atheroma plaque [34]. In combination, these data strongly suggest that the changes in galectin-3 distribution in the arteries of patients with PAD are a component of the molecular mechanisms underlying the plaque formation, and its progression. A caveat of the present study is that we cannot guarantee that the storage of the samples has not in any way altered the histological structure of the control or diseased arteries. This possibility is unlikely but it cannot be completely ruled out.

In contrast to the above, in performing quantitative analyses of galectin-3 expression, we found no significant differences between normal and PAD arteries. This could have resulted from the great difficulty in obtaining normal arteries. The samples we had derived from tissue donors who were victims of a traffic accident. Selection for the present study followed histological analysis to exclude the presence of atherosclerosis. This resulted in lowering of the number of samples available, and the statistical analysis less than reliable. However, the

confidence interval of galectin-3 staining measurements in patients with PAD covered the values observed in normal arteries. The postulation is that quantitative differences in expression between groups, if any, are not of high importance.

Alterations in plasma galectin-3 concentrations in PAD have been investigated by two independent research groups. Casanegra *et al.* [35] analyzed galectin-3 in 29 patients attending the Mayo Clinics with no evidence of PAD (normal ankle brachial index; ABI) and 31 patients with PAD (low ABI). They observed a mean galectin-3 concentration of 14.4 ng/mL in subjects with normal ABI, and approximately 22% increase in PAD patients. Our results are in the same range of measurement, albeit with some slight differences. Our values in the control group are lower, and the percentage increase in the patient group is approximately 76%. Demographic variation may account for these differences. In addition, the presence of concomitant diseases that could increase galectin-3 levels cannot be ruled-out in the control subjects of the Mayo Clinics' study. Moreover, the PAD populations studied were different in that we had a higher frequency of patients with resting pain, ulceration or gangrene. Madrigal-Matute *et al.* [7] reported plasma galectin-3 concentrations ranging between 2 and 20 ng/mL in PAD patients, and with no significant difference with respect to the severity of the disease. This finding is concordant with our observation that plasma galectin-3 concentrations are similar in patients with moderate or severe PAD. They also observed that concentrations above the median were significantly associated with an increase in total mortality risk. A finding of note from our present study is the lack of association between plasma galectin-3 concentrations and the presence of derangements such as arterial hypertension, diabetes, dyslipidemia, ischemic heart disease, or chronic

obstructive pulmonary disease. A possible explanation could be that the interaction of PAD and galectin-3 is stronger than in these other diseases and, as such, masking the influence on the circulating levels of galectin-3. In addition, our patients had been receiving protracted treatment with numerous medications, and this can also influence plasma galectin-3 concentrations in these concomitant diseases.

We observed direct correlations between plasma galectin-3 levels and markers of oxidative stress and inflammation, such as F2-isoprostanes, CCL2, CRP and B2M. Galectin-3 was shown [36] to induce oxidative stress through the release of $O_2^{\cdot -}$ in cultured mast cells, an effect that was blocked by the antioxidant enzyme superoxide dismutase. Galectin-3 is also expressed in human monocytes and released under NADPH oxidase-dependent superoxide synthesis [7]. In Wistar rats fed with a high-fat diet [37], leptin increased $O_2^{\cdot -}$ production by a mechanism that requires galectin-3. A recent prospective study in patients with chronic heart failure [38] showed direct correlations between galectin-3, markers of oxidative stress (oxidized low-density lipoproteins and extracellular superoxide dismutase), and markers of inflammation (CRP, interleukin-6) or heart failure (N-terminal pro b-type natriuretic peptide). These findings are similar to those in the present investigation.

Several studies [18,35] have suggested that the measurement of plasma galectin-3 concentrations may be a good biomarker of diseases related to atherosclerosis. The results from the present investigation show that, in PAD, the diagnostic accuracy of this parameter, albeit quite high, is not superior to that of CRP and B2M, and lower to that of F2-isoprostanes and CCL2. The efficacy of F2-isoprostanes and CCL2 in discriminating between healthy individuals and PAD patients has already been reported by our group [27], and there are no other biochemical

parameters identified to date that are superior in diagnostic accuracy. However, the ratios of galectin-3/CRP and galectin-3/B2M also showed a very high efficacy. Also, these measurements have the advantage over F2-isoprostanes and CCL2 in that they can be easily automated and implemented in routine Clinical Chemistry laboratories at a low cost and a speed of analysis [39-41]. Studies in wider series of patients need to be conducted to confirm the clinical usefulness of these ratios as PAD biomarkers.

4. Materials and Methods

4.1. Ethics approval

The Hospital's Ethics Committee (Institutional Review Board) approved the procedures of the study (approval documents 10-04-29/4proj3 and 2011-10-27/10proj1), and written informed consent was obtained from all the participants.

4.2. Clinical assessment of PAD severity

The extent of PAD was determined using the Fontaine classification, which defines 4 stages: Stage I, asymptomatic; Stage II, intermittent claudication; Stage III, rest pain; Stage IV, ulceration or gangrene [42]. We also employed the ankle-brachial index (ABI), defined as the ratio of the systolic blood pressure at the ankle to that in the upper arm. Compared to the arm, lower blood pressure in the leg is an indication of blocked arteries due to PAD [43].

4.3. Participants

For the histological and immunohistochemical study we analyzed portions of femoral or popliteal arteries from patients, obtained during

surgical procedures for infra-inguinal limb revascularization in the Vascular Surgery Department of *Hospital Universitari Joan XXIII* between January 2014 and June 2016 (*n* = 50). All samples were from patients at Stages III and IV of the Fontaine classification. Four normal arteries obtained from accident victims between March 2014 and August 2015, and stored at the Blood and Tissue Bank of Catalonia (Banc de Sang i Teixits, www.bancsang.net/es/donants/donacio_teixits.html, Barcelona, Spain) were used as controls. All samples were stored at −80ºC until processed for histological examination.

For the biochemical study we underwent an observational, prospective, cross-sectional study in patients attending the Vascular Surgery Department of *Hospital Universitari Joan XXIII*. Diagnosis of PAD was performed by measuring the ABI, together with non-invasive imaging and angiography, when indicated. We recruited 86 patients with symptomatic PAD (79.1% men, 42-89 years old), 95% having an ABI between 0.4 and 0.9, and 5% with an ABI lower than 0.4. Degree of PAD was determined using the Fontaine classification; 2.6%. of the patients were classified as Stage I; 43.6% as Stage II; 10.3% as Stage III; and 43.6% as Stage IV. Patients with clinical or analytical evidence of infection, acute ischemia, renal failure, liver disease, cancer or autoimmune diseases were excluded. Diabetes, hypertension and dyslipidemia were defined according to established criteria.

The control group was composed by 72 plasma samples obtained from healthy volunteers participating in a population-based study (65.3% men, 58-79 years old). Participants in this study were randomly drawn from the local government census of three communities in the Mediterranean region of Tarragona (N.E. Spain). All members of the control group underwent a physical examination and a blood test. They

could walk without any problems or pain, and were ostensibly healthy with no clinical or analytical evidence of infectious disease, renal insufficiency, hepatic damage, neoplasia, oligophrenia, or dementia. Their serum concentrations of CRP and B2M were within the normal range. Medication intake was not an exclusion criterion except in the case of drugs interfering with vitamin metabolism (methotrexate, tuberculostatics, theophylline, or vitamin B6 antagonists). The population studied did not consume vitamin supplements or local food fortified with vitamins. Pregnant or recent post-partum women were not included in the study. All plasma and serum samples were collected between 2014 and 2016 and stored at –80ºC in our Biological Sample Bank.

4.4. Histological and immunohistochemical study

Arteries were rinsed in phosphate buffer to remove residual blood and placed in at least 10 volumes of buffered formalin using a standard protocol for embedding tissue in paraffin wax for subsequent histology slide preparation. Three sections per slide were used for histological and immunohistochemical analyses. Sections, of 4-µm thickness, were stained with hematoxylin-eosin for histology. The *intima* and *media* thicknesses were measured in all histological sections as an estimate of the extent of atherosclerosis. The immunohistochemical expression of galectin-3 was analyzed using goat antibodies against human galectin-3 (dilution of 1/400) from R&D Systems, Inc. (Minneapolis, USA). The appropriate biotinylated secondary antibodies, (Vector Laboratories Inc., Burlingame, CA, USA) were used at a dilution of 1:200. Detection was performed with the ABC peroxidase system (Vector Laboratories, Burlingame, CA, USA) and 3,3'-diaminobenzidine (DAB) peroxidase substrate (Dako). All immunohistochemical sections were counterstained

with Mayer's hematoxylin. The positively-stained areas were quantified automatically (AnalySIS image software system),using an image analysis software (Soft Imaging System, Munster, Germany) and expressed as percentages of the total area. Control tissue samples were processed identically to the test samples except that the primary antibodies were omitted from the incubation. The immunohistochemical expressions of CD68 antigen and α-actin were used as a markers of macrophages and smooth muscle cells, respectively, and analyzed as previously reported [32,44].

4.5. Biochemical assessments

Concentrations of galectin-3 were measured in the ethylene diamine tetraacetate (EDTA)-plasma using enzyme immunoassay (R&D Systems®, Minneapolis, USA); Serum F2-isoprostanes and CCL2 were determined by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI, USA, and Prepotech, London, UK, respectively). Serum high-sensitivity CRP and B2M concentrations were measured by automated immunoturbidimetry (Roche Diagnostics, Mannheim, Germany) in a Roche Modular Analytics P800 system (Roche Diagnostics, Basel, Switzerland).

4.5. Statistical analyses

Differences between any two groups were assessed with the χ^2 test (categorical) or the Mann-Whitney *U* test (continuous) since most of the variables studied had non-parametric distributions. Spearman correlation coefficient was used to evaluate the degree of association between variables. The diagnostic accuracy of the measured biochemical variables was assessed by receiver operating characteristics (ROC) curves. This analysis represents plots of all the sensitivity/specificity pairs resulting

from varying decision thresholds. Sensitivity (or true positive rate) is the proportion of the sample correctly identified as being specific to the disease. Specificity (or true negative rate) is the proportion of subjects correctly identified as not being specific to the disease. False positive rate is calculated as 1-specificity. The area under the curve (AUROC) and 95% confidence interval (CI) was calculated. The AUROC represents the ability of the test to correctly classify patients, with respect to the investigated parameter alteration. The values of AUROC can range between 1 ("perfect" test) and 0.5 ("worthless" test) [45].

5. Conclusions

We observed differences in the expression of galectin-3 in normal arteries and arteries of patients with PAD, with a displacement of the expression from the *adventitia* to the *media* and the *intima* which may suggest involvement of galectin-3 in the site of the atherosclerosis plaque formation. In addition, plasma galectin-3 concentration was increased in PAD patients and correlated with serologic markers of oxidative stress and inflammation. The measurement of galectin-3 had a similar diagnostic accuracy to that CRP and B2M in the diagnosis of PAD. However, ratios of galectin-3/CRP and galectin-3/B2M showed improved usefulness as biomarkers in PAD.

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Author Contributions: Anna Hernández-Aguilera and Vicente Martín-Paredero were responsible for patient management and clinical data collection; Isabel Fort-Gallifa, and Anna Hernández-Aguilera took responsibility for the biological samples and the database; Isabel Fort-Gallifa, Anna Hernández-Aguilera, Anabel García-Heredia, Noemí Cabré, Fedra Luciano-Mateo, and Josep M. Simó performed the biochemical and histological analyses; Isabel Fort-Gallifa, Jorge Joven and Jordi Camps performed the statistical analyses and interpreted the results; Jordi Camps wrote the manuscript, which was subsequently discussed and modified by the rest of the team. All the authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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DISCUSIÓN

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Actualmente los marcadores serológicos utilizados en el cribado y pronóstico de la EAP son los mismos que se aplican en las otras ECV, tratándose fundamentalmente de marcadores de inflamación y riesgo trombótico. Entre los métodos utilizados para la valoración de la inflamación se encuentran la B2M y la PCR (determinada mediante el método de alta sensibilidad), tal y como recomiendan las guías europeas de la prevención de las ECV de 2016 (57).

El estudio de las ECV, y concretamente de la EAP, que es la patología que nos ocupa, ha permitido establecer una relación directa e indirecta entre la inflamación y la desregulación en los procesos pro-oxidantes y antioxidantes (49); esta situación es debida a que el estrés oxidativo, producto de esta desregulación, incrementa la expresión génica de moléculas inflamatorias (117).

En nuestro organismo la producción de ROS es el producto directo del propio metabolismo celular y también se generan como respuesta a factores ambientales, por esta razón la homeostasis del estrés oxidativo es una condición fisiológica que se mantiene en equilibrio en ausencia de patología (23). Tal y como se ha citado en la introducción, la aterosclerosis conlleva asociada una desregulación, transitoria o permanente, entre los mecanismos pro-oxidantes y antioxidantes implicados, tanto endógenos como exógenos, en nuestro organismo. A esta situación se la conoce como estrés oxidativo (118, 119).

La medición directa de ROS resulta muy difícil debido a la elevada reactividad de estas moléculas, característica que les confiere una muy corta vida media. Los métodos de medición requieren instrumentación poco extendida en los laboratorios clínicos actuales y poseen una especificidad limitada (120). Por esta razón la medición directa del estrés oxidativo mediante las ROS resulta dificultosa y su aplicación en la clínica asistencial prácticamente inviable (121). Como consecuencia de esta condición, el uso de la medición indirecta del estrés oxidativo mediante el cálculo de la concentración o de la actividad de moléculas endógenas modificadas por la oxidación es el método indirecto más utilizado para su evaluación (122).

Las moléculas utilizadas para la valoración indirecta del estrés oxidativo son DNA, RNA, carbohidratos, proteínas y lípidos (119). Estas moléculas sufren modificaciones causadas por la reacción de las ROS con su estructura nativa, confiriéndoles una característica medible y diferenciable de la molécula intacta.

Las aplicaciones de la medición de los biomarcadores de estrés oxidativo son básicamente tres, la evaluación del estrés oxidativo, la localización de los componentes redox en los procesos fisiológicos y patológicos y, finalmente, y la que ocupa esta disertación, la estimación de la severidad y progresión de una enfermedad (122).

La evaluación de la EAP mediante los biomarcadores establecidos proporciona una valoración indirecta de la extensión del daño aterosclerótico debido a su capacidad para evaluar el grado de inflamación y, en consecuencia, permiten estimar, *grosso modo*, la carga de estrés oxidativo, debido a que éste incrementa la expresión de las moléculas inflamatorias. En cambio, la valoración del estrés oxidativo, mediante los métodos indirectos citados anteriormente, nos ofrece una evaluación más minuciosa de la extensión de esta enfermedad independientemente del grado de inflamación e, incluso, si ésta todavía no está presente o no es lo suficientemente notable.

Nuestros hallazgos están de acuerdo con estudios previos que documentan un aumento del estrés oxidativo en pacientes con EAP (33, 123-125). Se considera a los F2-isoprostanos como el *gold standard* para evaluar la lesión oxidativa *in vitro* (76). Nuestros pacientes tenían, como promedio, concentraciones plasmáticas de F2-isoprostanos 10 veces superiores al grupo control. Estos resultados se observaron desde los primeros estadios de la enfermedad (Grados Fontaine I-II). Un aumento de F2-isoprostanos en pacientes con EAP fue descrito previamente por Mueller et al. (123), aunque, a pesar de ello, no se llegó a implementar la medición de F2-isoprostanos como biomarcador clínicamente relevante de la enfermedad, probablemente porque las diferencias reportadas entre sus pacientes y su población sana fueron menores que las observadas, por ejemplo, en la presente investigación. De hecho, nuestro estudio encontró curvas ROC con una excelente sensibilidad y

especificidad clínicas, y el análisis multivariante mantuvo sólo los F2-isoprostanos como un predictor independiente de EAP, mientras que excluyó la PCR, B2M y todas las otras variables medidas. Estudios recientes también hallaron concentraciones plasmáticas aumentadas de isoprostanos en pacientes con EAP. Berent et al. (126) describieron un aumento de los niveles de 8-epi-prostaglandina F2α en 100 pacientes con EAP, y Loffredo et al. (127) observaron altos niveles séricos de prostaglandina F2αIII en pacientes con EAP en estadio IIb de Fontaine, niveles que disminuyeron después del consumo de chocolate negro rico en polifenoles antioxidantes. Sólo hay un estudio que reporta hallazgos contradictorios. Signorelli et al. (128) encontraron disminución de los niveles de F2-isoprostanos en un pequeño grupo de pacientes con una ABI ≤ 0,9 junto con aumentos significativos en microRNAs (miR) miR-130, miR-210 y miR-27b, capaces de regular la expresión génica. Los autores interpretaron estos hallazgos como una respuesta adaptativa en estos pacientes para reducir el estrés oxidativo (es decir, la regulación positiva de las enzimas antioxidantes).

La presente Tesis Doctoral confirma el aumento de la concentración plasmática de CCL2 y la disminución de las actividades y concentración de PON1 en suero de pacientes con EAP (91, 96). Hemos descrito anteriormente que la determinación del cociente entre CCL2 y PON1 segrega los pacientes con EAP de los controles casi perfectamente (96). La respuesta inflamatoria, probablemente secundaria al estrés oxidativo aumentado, es extrema en los pacientes con EAP, lo que sugiere que estas alteraciones están causalmente relacionadas con la progresión de la enfermedad. Razonamos que los pacientes con EAP representan un modelo clínico con un considerable grado de enfermedad de múltiples vasos y alta carga de aterosclerosis. Los resultados obtenidos de la medición de las concentraciones de CCL2 y PON1, así como las actividades de PON1 paraoxonasa y lactonasa, confirman nuestra hipótesis y pueden constituir nuevos indicadores del estado de la enfermedad. En nuestro estudio, la disminución de las actividades de PON1 se asociaron con un aumento de las concentraciones de CCL2. Sin embargo, esta relación inversa no se confirmó a nivel de tejido. De hecho, los estudios anteriores de nuestro grupo mostraron que la expresión de ambas proteínas está aumentada en las arterias de los pacientes con EAP (81).

Esta observación sugiere que las variaciones en los niveles circulantes de PON1 y CCL2 no necesariamente se correlacionan con su posible papel a nivel celular. Tal vez la expresión de la proteína PON1 se incrementa en las arterias enfermas para contrarrestar el estrés oxidativo y la inflamación inducida por CCL2. Sin embargo, esta hipótesis debe ser confirmada por investigaciones adicionales.

Los resultados del segundo estudio muestran diferencias importantes en la distribución de galectina-3 en arterias normales y arterias de pacientes con EAP. Las arterias normales mostraron expresión de galectina-3 exclusivamente en la túnica adventicia, mientras que las arterias de los pacientes mostraron la localización principalmente adyacente a las células del músculo liso de la túnica media, con una pequeña tinción en adventicia y en los casos de placa aterosclerótica avanzada expresaron tinción en íntima. La interpretación fisiopatológica de este hallazgo no puede deducirse de la presente investigación, pero datos previos sugieren que esta distribución diferencial podría estar relacionada con los procesos de inflamación, fibrogénesis y calcificación de la placa aterosclerótica. De hecho, los pacientes con una placa avanzada expresaron galectina-3 junto con macrófagos en la túnica íntima, lo que sugiere un papel coordinado en la formación de la placa. Esta hipótesis es apoyada por datos que demuestran que la galectina-3 estimula la activación de miofibroblastos y la fibrosis en varios tipos de células (129, 130). En las válvulas aórticas de pacientes con estenosis aórtica, la galectina-3 co-localiza con los marcadores de músculo liso actina y vimentina y con marcadores osteogénicos tales como osteopontina, y la proteína 2 morfogenética ósea (131). Varios estudios indican que las placas de la carótida humana expresan galectina-3 en las células del músculo liso, especialmente en las zonas macrocalcificadas donde se co-localiza con la fosfatasa alcalina (132). La conclusión es que, bajo ciertos estímulos, las células del músculo liso arterial pueden desplazar sus fenotipos a un estado parecido a los macrófagos (133). Además, otro estudio de nuestro grupo mostró una distribución similar de CCL2 y sus receptores en la íntima de los pacientes con EAP avanzada y placa de ateroma (81). En conjunto, estos datos sugieren fuertemente que los cambios en la distribución de galectina-3 en las arterias de pacientes con

EAP son un componente de los mecanismos moleculares subyacentes a la formación y progresión de la placa de ateroma.

Dos grupos de investigación independientes han investigado las alteraciones en las concentraciones plasmáticas de galectina-3 en la EAP. Casanegra et al. (134) analizaron galectina-3 en 29 pacientes que acudieron a la Clínica Mayo y que no tenían evidencia de EAP (ABI normal) y en 31 pacientes con EAP (ABI bajo). Observaron una concentración media de galectina-3 de 14,4 ng/ml en sujetos con ABI normal y un aumento de aproximadamente 22% en pacientes con EAP. Nuestros resultados están en el mismo rango de medida, aunque con algunas ligeras diferencias; nuestros valores en el grupo de control son más bajos, y el porcentaje de aumento en el grupo de pacientes es de aproximadamente el 76%; variaciones demográficas puede explicar estas diferencias. Además, la presencia de enfermedades concomitantes que podrían aumentar los niveles de galectina-3 no pueden descartarse en los sujetos control del estudio de Casanegra et al. Asimismo, las poblaciones de pacientes estudiadas fueron diferentes, ya que en nuestro estudio existía una mayor frecuencia de pacientes con dolor de reposo, ulceración o gangrena. Madrigal-Matute et al. (101) reportaron concentraciones plasmáticas de galectina-3 entre 2 y 20 ng/ml en pacientes con EAP, sin encontrar diferencias significativas con respecto a la gravedad de la enfermedad. Este hallazgo es concordante con nuestra observación en que las concentraciones plasmáticas de galectina-3 son similares en pacientes con EAP moderada o grave. También observaron que las concentraciones por encima de la mediana se asociaron significativamente con un aumento en el riesgo de mortalidad total.

Los resultados de la presente investigación sugieren que los F2-isoprostanos y el CCL2 tienen un valor predictivo superior a la PCR y la B2M en el diagnóstico de EAP, especialmente en las primeras etapas de la enfermedad. Varios estudios describieron que las concentraciones séricas de PCR o de B2M están elevadas en pacientes con EAP, y que los valores se correlacionan con el ABI y la función endotelial (63, 66, 135-137).

Sin embargo, las elevaciones encontradas fueron moderadas y, por lo tanto, se propusieron paneles de varios biomarcadores para mejorar su precisión diagnóstica. Uno de estos paneles incluía cistatina C, PCR, B2M y glucosa, pero a pesar de ello, el poder discriminador no fue muy elevado (138). Otro estudio propuso la inclusión del marcador genético rs10757269, un polimorfismo en el cromosoma 9p21. Cuando se añadió a B2M, cistatina C y PCR, el poder discriminatorio del panel aumentó considerablemente. Sin embargo, el principal inconveniente de esta propuesta es que los marcadores genéticos pueden estar influenciados por la etnia, y este tema no ha sido suficientemente investigado en el caso del polimorfismo rs10757269 (139). Otro panel propuesto incluyó 9 biomarcadores diferentes: PCR, interleucina-6, receptor II del factor de necrosis tumoral, lipoproteína (a), péptido natriurético cerebral, péptido natriurético auricular, arginina vasopresina, osteoprotegerina y fibrinógeno (140). Estos paneles múltiples son costosos y difíciles de implementar en la práctica clínica estándar. Por el contrario, nuestro estudio sugiere que la medición de un solo parámetro, los F2-isoprostanos o el CCL2, segrega realmente y casi perfectamente, los pacientes con EAP de la población sana.

Los resultados de la presente investigación muestran que en la EAP, la precisión diagnóstica de la determinación de galectina-3, aunque bastante alta, no es superior a la de la PCR y B2M, e inferior a la de los F2-isoprostanos y CCL2. La eficacia de F2-isoprostanos y CCL2 en la discriminación entre los individuos sanos y los pacientes con EAP es difícilmente mejorable, aunque los cocientes galectina-3/PCR y galectina-3/B2M también mostraron una eficacia muy alta. Además, estas mediciones tienen la ventaja sobre F2-isoprostanos y CCL2 en que pueden ser fácilmente automatizadas e implementadas en los laboratorios de química clínica a un bajo costo y una alta velocidad de análisis (141-143).

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CONCLUSIONES

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1.- Los pacientes con EAP presentaron un importante aumento de las concentraciones circulantes de F2-isoprostanos y CCL2 comparados con el grupo control.

2.- Se observaron diferencias en la expresión de galectina-3 en las arterias normales y en las arterias de los pacientes con EAP, con un desplazamiento de la expresión de la adventicia a la media y la íntima, lo que puede sugerir la participación de galectina-3 en la formación de la placa de ateroma.

3.- La concentración de galectina-3 plasmática aumentó en pacientes con EAP y se correlacionó con marcadores serológicos de estrés oxidativo e inflamación.

4.- Las mediciones de las concentraciones circulantes de F2-isoprostanos y CCL2 mostraron una excelente capacidad de discriminación entre los sujetos sanos y los pacientes con EAP, lo que sugiere que pueden ser buenos candidatos a biomarcadores de esta enfermedad.

5.- La determinación de la concentración plasmática de galectina-3 tuvo una precisión diagnóstica similar a la PCR y B2M en el diagnóstico de EAP. Sin embargo, el cociente galectina-3/PCR mostró una precisión mejorada y sólo algo inferior a las de los F2-isoprostanos y CCL2, con la ventaja adicional de su fácil automatización en el laboratorio clínico.

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MATERIAL SUPLEMENTARIO

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Original article

Biochemical indices of oxidative stress and inflammation in the evaluation of peripheral artery disease

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ABSTRACT

Background: The aims of this study were: (1) to investigate changes in indices of oxidative stress and inflammation in the evaluation of peripheral artery disease (PAD); (2) to compare the diagnostic efficacy of these parameters with that of classical clinical laboratory routine parameters.
Design and methods: We studied 115 patients with PAD and 300 healthy volunteers.
Results: PAD patients had significantly increased circulating concentrations of F_2-isoprostanes, protein carbonyls, chemokine (C-C motif) ligand 2 (CCL2), high-sensitivity C-reactive protein (hs-CRP), β-2-microglobulin (B2M), and decreased paraoxonase-1 (PON1) levels. When patients were classified according to the Fontaine score, we observed important increases in plasma F_2-isoprostanes and CCL2 that appeared in milder stages of the disease, and remained so at similar levels in more advanced stages; almost no overlapping with the control group was noted. Receiver operating characteristics analysis comparing patients and controls revealed that the areas under the curve for F_2-isoprostanes and CCL2 approached unity [0.999 (0.998–1.000) and 0.993 (0.985–1.000)], respectively, and significantly higher to those of the other measured parameters.
Conclusion: Our data suggest that F_2-isoprostanes and CCL2 measurements may be useful tools for the diagnosis of PAD.

1. Introduction

Lower-extremity peripheral artery disease (PAD) is an important manifestation of atherosclerosis that is associated with severe impairment of different arterial territories. Indeed, PAD is a predictor of coronary and cerebral vascular disease risk [1]. The disease prevalence increases with age and, in people over the age of 70 years, it is estimated to be about 20% [2]. Inflammation is important for the initiation and progression of PAD, and the inflammatory mediators involved in this process are similar to those contributing to coronary artery disease. Smoking and diabetes mellitus, the strongest predictors of PAD, promote oxidative stress, which enhances inflammatory pathways [3]. Atherosclerosis affects wide portions of arteries in the lower extremities of PAD patients, resulting in progressive functional impairment and decline, and greatly reducing quality-of-life. This deterioration is the effect of a silent progression of the disease and, as such, appropriate and effective prevention measures are applied too late, or not implemented at all [2,4,5]. Moreover, asymptomatic PAD is several times more common in the general population than the symptomatic disease [6]. Hence, the search of biomarkers to enable early diagnosis of the disease, or the prediction of future complications, is an active line of research. Several studies have proposed C-reactive protein, measured by a high-sensitivity method (hs-CRP), or β-2-microglobulin (B2M) as useful markers of PAD. However, their use in the diagnosis of this disease is still an unresolved issue [7,8].

Oxidative stress is implicated in the development of

Abbreviations: ABI, ankle–brachial index; AUROC, areas under the curve of the ROC plots; B2M, β-2-microglobulin; CCL2, chemokine (C-C motif) ligand 2; CRP, C-reactive protein; EDTA, ethylene diamine tetraacetate; ELISA, enzyme-linked immunosorbent assay; GC/MS, gas chromatography/mass spectrometry; HDL, high-density lipoproteins; PAD, peripheral artery disease; PHC, primary health centers; PON, paraoxonase; ROC, receiver operating characteristics; SPSS, statistical package for social sciences; TBBL, 5-thiobutyl butyrolactone

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atherosclerotic vascular diseases, including PAD [9]. Several studies have reported that oxidized low-density lipoprotein uptake by macrophages promotes, among other responses, an inflammatory reaction that increases the production of the inflammatory chemokine (C-C motif) ligand 2 (CCL2). The consequence is the stimulation of arterial fatty streak formation and atheroma [10]. Oxidative stress can be evaluated in the circulation through the measurement of several markers such as lipid peroxidation, protein carbonylation or DNA damage. Isoprostanes are products of arachidonic acid and other polyunsaturated fatty acids. Similar to prostaglandins, they are generated *via* free-radical catalyzed mechanisms [11]. The subclass composed of F_2-isoprostanes, is one of the most abundant and, of these, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) measurement is considered an accurate method for evaluating oxidative stress in humans [12]. PAD patients have increased serum 8-iso-$PGF_{2\alpha}$, and this parameter is seen as an independent predictor of the disease [13]. In contrast, data on oxidative damage to proteins are scarce. Among the numerous products of oxidative damage of proteins, measurement of protein carbonyls is the most widely used type of protein damage for inferring oxidative stress. Specific protein carbonylations are thought to have clinical significance, since they function as biological signals of irreversibly altered protein structure and function [14]. Conversely, oxidative stress is counteracted by several antioxidant systems, one of the most important being the paraoxonase (PON) enzyme system. These antioxidant enzymes protect lipoproteins and cells from peroxidation and, as such, can mitigate the atherosclerosis process and vascular diseases [15]. The PON family contains three enzymes: PON1, PON2 and PON3, the genes of which are located adjacent to each other on chromosome 7q21–22. PON1 and PON3 are found in many tissues, as well as in blood, where they are associated with high-density lipoproteins (HDL). Conversely, PON2 is exclusively intracellular [15]. The most extensively studied among the PON enzyme family is PON1 because alterations in the structure and concentration reflect pathological status. Also, it is the most abundant in the circulation and can be analyzed by simple laboratory methods. Clinical data suggest that circulating serum PON1 activity may be an important marker of a variety of diseases that involve an inflammatory response to an increased oxidative stress, including PAD [16–18].

The aims of the present study were: (1) to investigate changes in F_2-isoprostanes, protein carbonyls, PON1, and CCL2, in the progression of PAD; (2) to compare the diagnostic efficacy of these parameters with that of hs-CRP and B2M.

2. Materials and methods

2.1. Ethics approval

The Hospital's Ethics Committee (Institutional Review Board) approved the procedures of the study (approval documents 10-04-29/4proj3 and 2011-10-27/10proj1), and written informed consent was obtained from the participants.

2.2. Participants

This is a prospective, observational, cross-sectional study in patients regularly attending the Vascular Disease Department of *Hospital Universitari Joan XXIII*. Diagnosis of PAD was performed with measurements that included the ankle–brachial index (ABI), non-invasive imaging, and angiography when indicated. We recruited 115 patients; 45 having an ABI between 0.4 and 0.9, and 70 with an ABI lower than 0.4. The Fontaine classification was used to evaluate the degree of PAD severity [19]. Participants with clinical or analytical evidences of infection, acute ischemia, renal failure, liver disease, cancer, or autoimmune diseases were excluded. Diabetes, hypertension and dyslipidemia were defined according to established criteria [20–22]. The control group was composed of 300 blood samples obtained from healthy volunteers participating in a population-based study. Participants of white-Mediterranean ethnic origin, were identified as being ostensibly healthy with no clinical or analytical evidence of infectious disease, renal insufficiency, hepatic damage, neoplasia, oligophrenia, or dementia. Thirty-three participants had hypertension (11.0%), 16 had dyslipemia (5.3%), and 11 had type II diabetes mellitus (3.7%). Medication intake was not an exclusion criterion except in the case of drugs interfering with vitamin metabolism (methotrexate, tuberculostatics, theophylline, or vitamin B_6 antagonists). The population studied did not consume vitamin supplements or local food fortified with vitamins. Pregnant women were not included in the study. The samples were stored at –80 °C in our Biological Sample Bank. A detailed description of this population has been published previously [23].

2.3. Biochemical measurements

The true physiological substrates for PON1 have not as yet been identified. Since PON1 has lactonase and esterase activities [15], we opted to analyze the catalytic activity of PON1 using two different substrates: 5-thiobutyl butyrolactone (TBBL, a synthetic lactone) and paraoxon (an ester), as previously described [16]. Briefly, TBBLase activity was measured in an assay containing 1 mM $CaCl_2$, 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 (1 U=1 mmol of TBBL hydrolyzed per minute). Serum PON1 paraoxonase activity was determined as 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 $CaCl_2$ [24]. Activities were expressed as U/L (1 U=1 µmol of paraoxon hydrolyzed per minute). Serum PON1 concentrations were determined by an in-house enzyme-linked immunosorbent assay (ELISA) with a rabbit polyclonal antibody generated against the synthetic peptide CRNHQSSYQTRLNALREVQ which is sequence specific for mature PON1 [25]. PON1 specific activities were calculated as the ratio between the activity and the corresponding concentration. The ethylene diamine tetraacetate (EDTA)-plasma concentrations of F_2-isoprostanes were determined by Enzyme Immunoassay (Cayman Chemical Co., Ann Arbor, Michigan, USA). Plasma protein carbonyl content was measured by a colorimetric assay (Cayman Chemical Co.). The hs-CRP concentration in serum was measured by automated turbidimetry (Roche Diagnostics, Mannheim, Germany). The serum concentrations of B2M and the EDTA-plasma concentration of CCL2 were measured by ELISA (Biovendor, Brno, Czech Republic, Roche Diagnostics, and Prepotech, London, UK, respectively). Serum cholesterol, HDL cholesterol, triglycerides, and insulin concentrations were analyzed by standard automated procedures in the Clinical Chemistry laboratory of *Hospital Universitari Joan XXIII*.

2.4. Statistical analyses

All calculations were performed with the statistical package for social sciences (SPSS 22.0, Chicago, IL, USA). Differences between any two groups were assessed with the χ^2 test (categorical) or the Mann-Whitney *U* test (continuous), since most of the studied variables had non-parametric distributions. Differences between more than two groups were assessed by the Kruskal-Wallis test. Spearman correlation coefficient was used to evaluate the degree of association between variables. The diagnostic accuracy of the measured biochemical variables was assessed by receiver operating characteristics (ROC) curves [26]. A binary logistic regression

analysis using the Wald forward step-by-step method was applied to identify independent predictors of PAD. The stepwise selection method is one in which the test-for-entry is based on the significance of the score statistics, and the test-for-removal is based on the lack of significance of the association between the putative predictor and the dependent variable. Candidate variables selected for logistic regression modeling were age, gender, smoking status, hypertension, diabetes mellitus, dyslipidemia, hs-CRP, B2M, F_2-isoprostanes, lactonase activity, PON1 concentration, and CCL2. Wald statistics were reported for variables in the final model. Unless otherwise indicated, results are shown as medians and 95% Confidence Interval (CI).

3. Results

3.1. Clinical characteristics of the PAD patients

A large majority of the patients were symptomatic, according to the Fontaine classification and most were at stages III and IV when recruited into the study. As expected, patients were older and the percentage of men was higher than in the control group. PAD patients were in receipt of several medications for the treatment of their disease. Intake of vitamins or mineral supplements was not recorded. The incidence of current habit of smoking and alcohol consumption was lower in the patients reflecting, perhaps, adherence to their physician's recommendations. Hypertension, diabetes mellitus, dyslipidemia, heart disease and pulmonary disease were common in PAD patients, but cerebrovascular events were not recorded (Table 1).

3.2. Selected biochemical variables are altered in PAD patients but they are not strongly related to the severity of the disease

Compared to the control group, PAD patients had significant increases in the circulating concentrations of triglycerides, insulin, hs-CRP, B2M, F_2-isoprostanes, protein carbonyls, and CCL2, together with decreases in PON1 lactonase and paraoxonase activities and PON1 concentrations. Since the decrease in serum PON1 concentration was higher than that of the enzyme activities, both lactonase and paraoxonase specific activities were significantly increased in patients with PAD (Table 1). When patients were classified according to the Fontaine score, we observed important increases in F_2-isoprostanes and CCL2 that appeared in milder stages of the disease, and remained at similar levels in more advanced stages. There was almost no overlap with the control group. Serum PON1 concentration and PON1 lactonase and paraoxonase activities showed a progressive decrease, albeit the degree of overlap was higher. Protein carbonyls and the standard tests (hs-CRP and B2M) significantly increased in relation to the Fontaine score but, in general, alterations appeared later to those F_2-isoprostanes or CCL2, and the degree of overlap among groups was considerable(Figs. 1 and 2).

As expected, we observed significant direct correlations between markers of oxidative stress and inflammation in PAD patients, and inverse correlations between these markers and PON1-related variables (Figs. 3 and 4).

Because the PAD patients and the control group were clearly different in terms of age and gender distribution, we investigated the influence of these characteristics on the analyzed parameters. Results are shown in Supplementary Tables 1 and 2. Among the investigated parameters none was influenced by gender, neither in the control group nor in the PAD patients. Only paraoxonase and lactonase activities were slightly influenced by age in the control group. Raw data for the PON-1 related variables and an example of the calibration curves for serum PON1 concentration measurements are shown in Supplementary Table 3 and Supplementary Fig. 1, respectively. Intake of medications did not produce any significant change in F_2-isoprostanes concentrations in PAD patients (Supplementary Table 4).

Table 1
Demographic, clinical, and biochemical characteristics of PAD patients and the control group.

Variable	Control group $n=300$	PAD $n=115$	p
Age, years	47 (18–80)	70 (50–92)	< 0.001
Male gender, *N* (%)	130 (43.3)	100 (86.9)	< 0.001
Smokers, *N* (%)	99 (33.0)	10 (8.7)	< 0.001
Alcohol consumption > 20 g/day, *N* (%)	75 (25.0)	8 (6.9)	< 0.001
Arterial hypertension, *N* (%)	31 (10.3)	79 (68.7)	< 0.001
Diabetes mellitus, *N* (%)	12 (4.0)	76 (66.1)	< 0.001
Dyslipidemia, *N* (%)	12 (4.0)	46 (40.0)	< 0.001
Ischemic heart disease, *N* (%)	0	28.6	< 0.001
Chronic obstructive pulmonary disease, *N* (%)	0	19.5	< 0.001
Fontaine classification score, *N* (%)			
Stage I, asymptomatic	N. A.	3 (2.6)	
Stage II, intermittent claudication		19 (16.5)	
Stage III, resting pain		27 (23.4)	
Stage IV, ulceration or gangrene		66 (57.4)	
Medications, *N* (%):			
Antiplatelet drugs	N. R.	34 (29.6)	
Statins		64 (55.6)	
Antidiabetic drugs		56 (48.7)	
Angiotensin converting enzyme inhibitors		56 (48.7)	
Angiotensin receptor antagonists		55 (47.8)	
Calcium receptor antagonists		74 (64.3)	
Diuretics		61 (53.0)	
Anti-arrhythmia drugs		98 (85.2)	
Beta-blockers		97 (84.3)	
Bronchodilators		79 (68.7)	
Cholesterol, mmol/L	5.2 (3.7–7.0)	3.9 (2.6–5.9)	< 0.001
HDL cholesterol, mmol/L	1.46 (0.96–2.20)	1.06 (0.59–1.79)	< 0.001
Triglycerides, mmol/L	1.1 (0.5–2.9)	2.9 (1.7–4.8)	< 0.001
Insulin, μmol/L	49.4 (20.1–125.3)	78.9 (15.1–423.7)	< 0.001
hs-CRP, mg/L	0.13 (0.01–1.42)	1.24 (0.09–4.67)	< 0.001
β-2-microglobulin, mg/L	1.40 (0.44–2.29)	2.52 (1.34–8.63)	< 0.001
F_2-isoprostanes, ng/L	8.59 (2.44–21.79)	90.91 (45.45–171.70)	< 0.001
Protein carbonyls, μmol/g	1.02 (0.54–2.00)	1.53 (0.74–2.82)	< 0.001
Lactonase, U/L	5.49 (3.20–8.84)	3.12 (1.17–6.27)	< 0.001
Paraoxonase, U/L	278.77 (161.30–579.90)	197.17 (84.07–473.19)	< 0.001
PON1 concentration, mg/L	96.46 (43.60–290.49)	27.10 (13.69–63.41)	< 0.001
Lactonase specific activity, U/mg	0.055 (0.016–0.133)	0.114 (0.026–0.264)	< 0.001
Paraoxonase specific activity, U/mg	2.90 (0.78–9.52)	5.82 (1.95–19.00)	< 0.001
CCL2, ng/L	138.52 (85.55–258.96)	586.50 (284.75–1185.00)	< 0.001

Except when otherwise indicated, results are shown as medians and 95% CI (in parenthesis).
CCL2: Chemokine (C-C motif) ligand 2; N.A.: Not applicable; N.R: Not recorded; PON1: Paraoxonase-1.

3.3. Diagnostic accuracy of the selected biochemical variables

When comparing the diagnostic accuracy of the selected

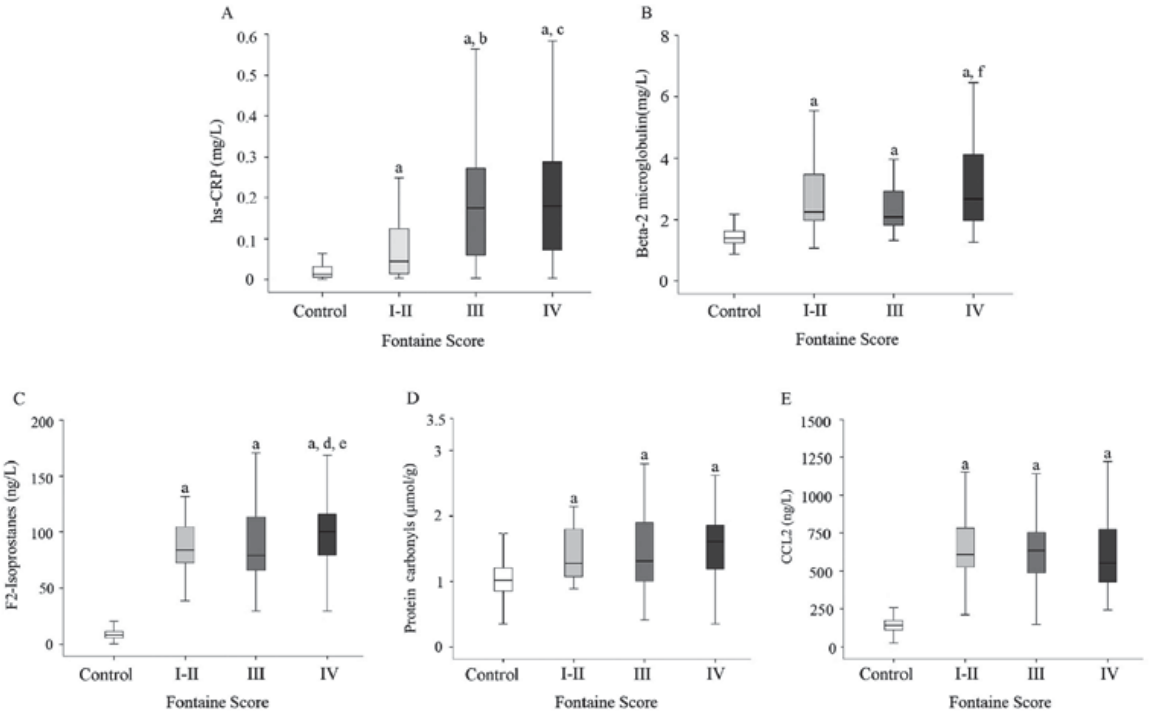


Fig. 1. Selected markers of oxidative stress and inflammation in PAD patients (classified according to the Fontaine score) and in the control group. Significance values by the Mann-Whitney *U* test: [a]$p < 0.001$, and [b]$p < 0.01$ with respect to the control group; [c]$p < 0.001$, and [d]$p < 0.05$ with respect to Fontaine stage I-II; [e]$p < 0.01$, and [f]$p < 0.05$ with respect to Fontaine stage III. Overall variations $p < 0.001$ by the Kruskal-Wallis test.

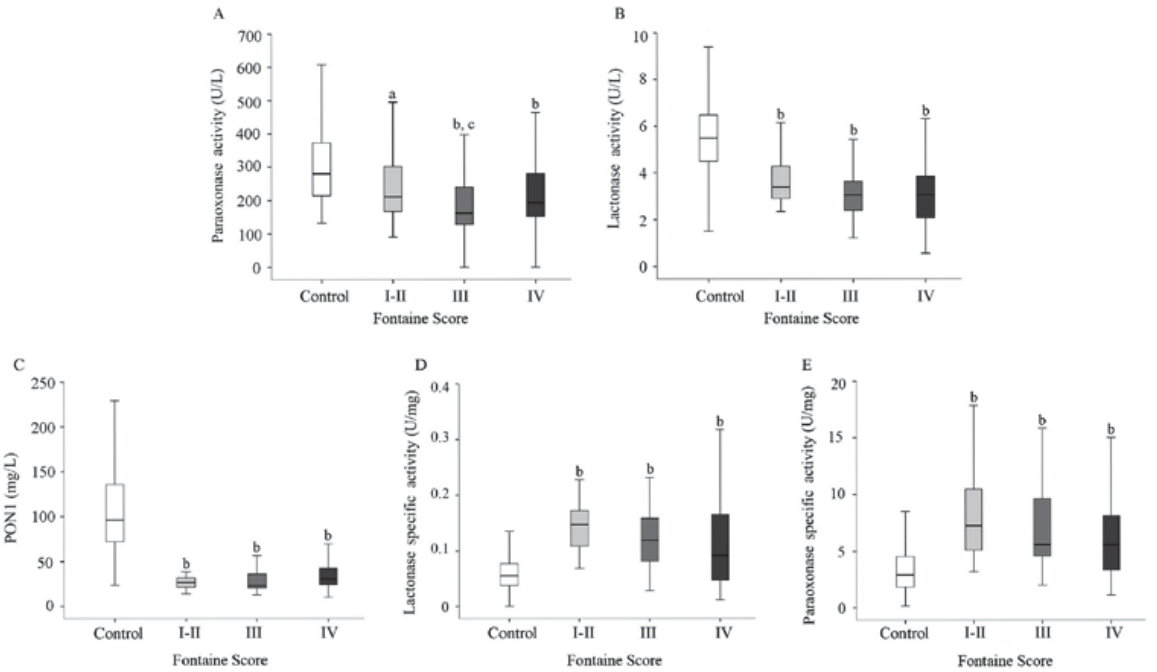


Fig. 2. PON1-related variables in PAD patients (classified according to the Fontaine score) and in the control group. Significance values by the Mann-Whitney *U* test: [a]$p < 0.01$, and [b]$p < 0.001$ with respect to the control group; [c]$p < 0.01$ with respect to Fontaine stage I-II. Overall variations $p < 0.001$ by the Kruskal-Wallis test.

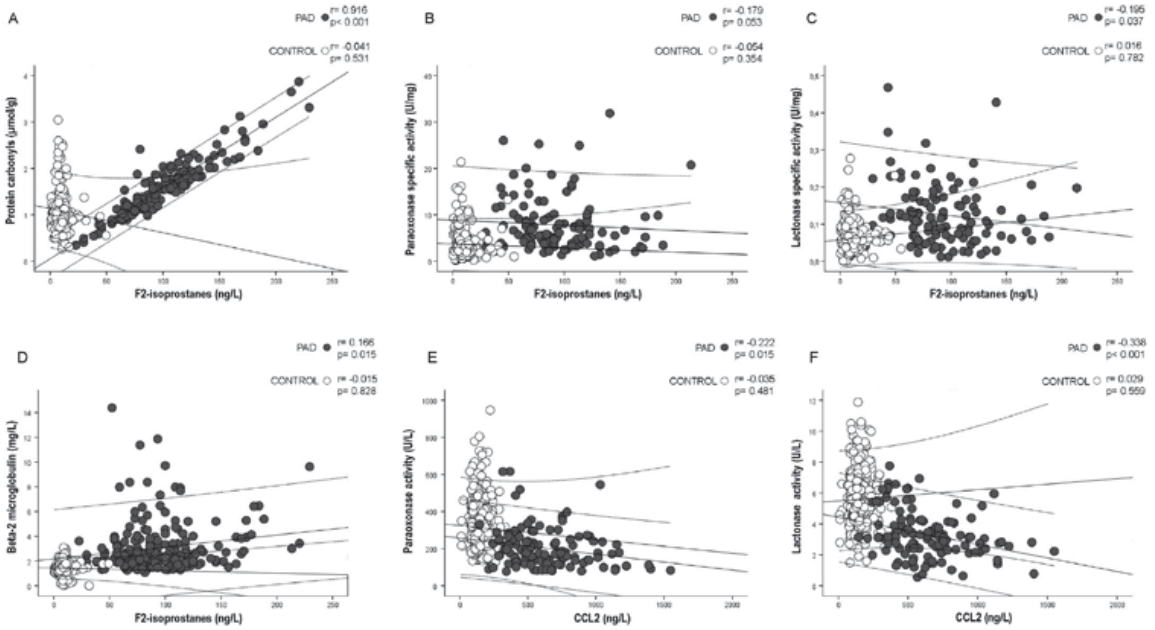


Fig. 3. Statistically significant correlations between selected biochemical variables. The continuous lines represent the regression lines, and the dashed lines, the 95% Confidence Intervals. Correlations calculated by the Spearman's ρ test.

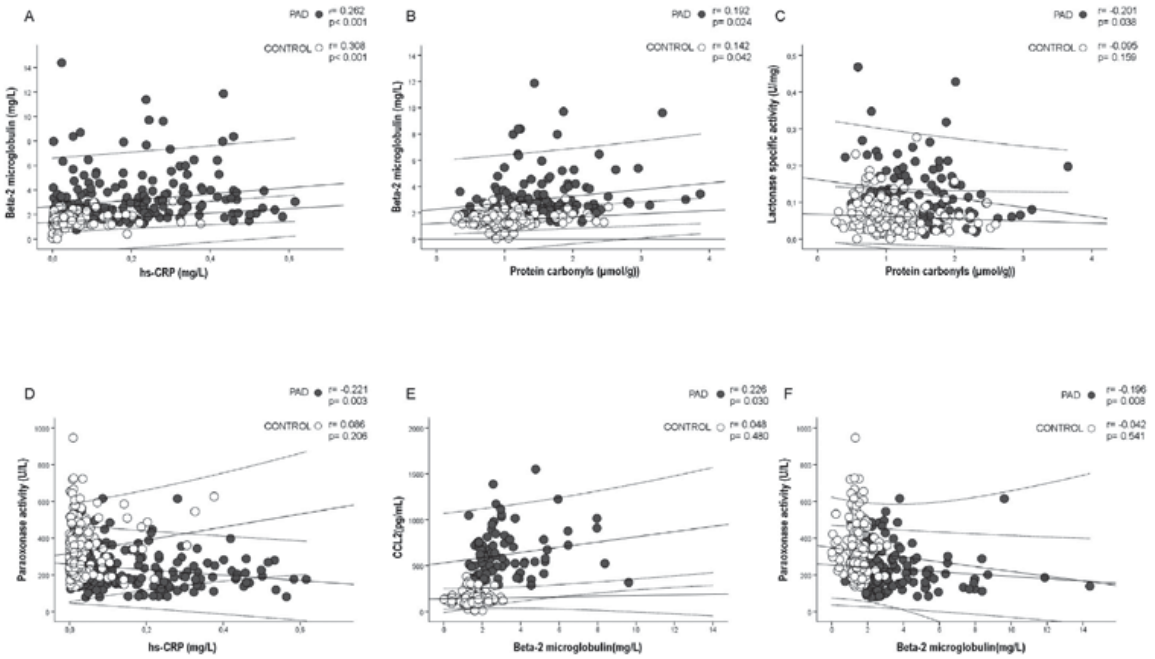
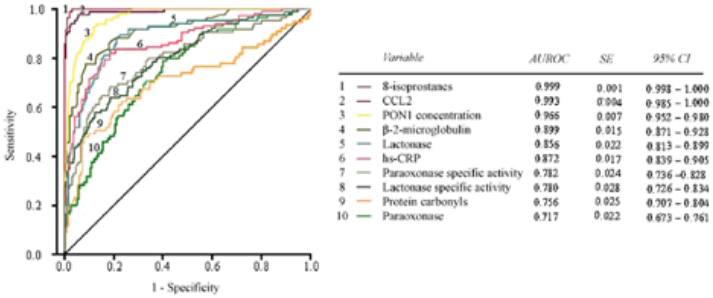


Fig. 4. Statistically significant correlations between selected biochemical variables. The continuous lines represent the regression lines, and the dashed lines, the 95% Confidence Intervals. Correlations calculated by the Spearman's ρ test.

variables in discriminating between the healthy volunteers and the patients with PAD, we found that the AUROC for F_2-isoprostanes and CCL2 were very close to unity, and significantly higher to those of the other measured parameters. The order of the calculated accuracies was: F_2-isoprostanes ≈ CCL2 > PON1 concentration > B2M > lactonase ≈ hs-CRP > paraoxonase



	Variable	AUROC	SE	95% CI
1	8-isoprostanes	0.999	0.001	0.998 – 1.000
2	CCL2	0.993	0.004	0.985 – 1.000
3	PON1 concentration	0.966	0.007	0.952 – 0.980
4	β-2-microglobulin	0.899	0.015	0.871 – 0.928
5	Lactonase	0.856	0.022	0.813 – 0.899
6	hs-CRP	0.872	0.017	0.839 – 0.905
7	Paraoxonase specific activity	0.782	0.024	0.736 -0.828
8	Lactonase specific activity	0.780	0.028	0.726 – 0.834
9	Protein carbonyls	0.756	0.025	0.707 – 0.804
10	Paraoxonase	0.717	0.022	0.673 – 0.761

Fig. 5. Receiver operating characteristics (ROC) plots for all the studied biomarkers in PAD patients, and in the control group. AUROC: areas under the curve of the ROC plots. SE: Standard Error.

specific activity ≈ lactonase specific activity ≈ protein carbonyls ≈ paraoxonase (Fig. 5).

3.4. Binary logistic regression analysis

Wald's forward binary regression analysis identified, in step 1, F_2-isoprostanes as the only biochemical variable significantly associated with PAD. Step 2 introduced CCL2, although the *p* value did not reach statistical significance. All the other investigated variables (demographical and clinical variables, PON1 concentration, B2M, lactonase, and hs-CRP) did not enter into the model (Table 2).

4. Discussion

According to current consensus, atherosclerosis represents a state of enhanced oxidative stress and inflammation characterized by lipid and protein oxidation in the vascular wall, together with increased synthesis of pro-inflammatory cytokines and chemokines. The oxidative modification hypothesis of atherosclerosis predicts that low-density lipoprotein oxidation is an early event in atherosclerosis and that oxidized low-density lipoproteins plays a key role in the onset and development of this disease. Changes include the production of reactive oxygen and nitrogen species by vascular cells, as well as oxidative modifications contributing to important clinical manifestations of artery disease, such as endothelial dysfunction [27]. Oxidative stress and inflammation are phenomena that have been extensively investigated in relation to coronary artery disease and myocardial infarction. However, its relationship with PAD is not as well documented. This is surprising since the clinical burden of atherosclerosis is much higher in PAD than in coronary disease which, *a priori*, suggests that alterations in the circulation are much higher and would correlate better with the disease.

Table 2
Binary logistic regression analysis of the variables associated with the presence of PAD.

		B	SE	Wald	*p*	Exp (B)	95% CI Exp (B)
Step 1	F_2-isoprostanes	0.211	0.039	29.6	< 0.001	1.235	1.145–1.333
	Constant	−7.384	1.256	34.59	< 0.001	0.001	
Step 2	CCL2	0.034	0.022	2.419	0.120	1.034	0.991–1.079
	F_2-isoprostanes	0.163	0.083	3.894	0.048	1.177	1.001–1.385
	Constant	−17.087	9.048	3.567	0.059	0.000	

Step 1: Cox and Snell r^2=0.668; Negelkerke r^2=0.957; −2 log of likelihood: 28.379.
Step 2: Cox and Snell r^2=0.693; Negelkerke r^2=0.992; −2 log of likelihood: 5.575.
All the other variables had been excluded from the model.

Our findings are in accordance with previous studies documenting enhanced oxidative stress in patients with PAD [13,28–30]. The F_2-isoprostanes have been reported to be the "gold standard" to assess oxidative injury *in vitro* [12]. Our patients had, on average, 10 times higher plasma concentrations of F_2-isoprostanes than the control group. These results were observed from the earliest stages of the disease (Fontaine stages I-II). An increase in F_2-isoprostanes in PAD patients had been reported 11 years ago by Mueller et al. [13] *i.e.* that 8-iso-$PGF_{2\alpha}$ was an independent predictor of PAD. However, despite their important findings, measurement of 8-iso-$PGF_{2\alpha}$ as a clinically-relevant biomarker of the disease has not been implemented, probably because the reported differences with their healthy population were lower than those observed, for example, in the present investigation. Indeed, we obtained ROC curves with an excellent clinical sensitivity and specificity, and multivariate analysis retained only F_2-isoprostanes as an independent predictor of PAD, while excluding hs-CRP, B2M and all the other measured variables. Very recent studies also found increased plasma concentrations of isoprostanes in PAD patients. Berent et al. [31] reported increased levels of 8-epi-prostaglandin $F_2\alpha$ in 100 patients with PAD, and Loffredo et al. [32] observed high levels of serum prostaglandin $F_2\alpha$III in PAD patients at Fontaine stage IIb, levels that decreased after consumption of dark chocolate rich in antioxidant polyphenols. There is only one study reporting contradictory findings. Signorelli et al. [33] found decreased levels of F_2-isoprostanes in a small group of patients with an ABI ≤0.9 together with significant increases in microRNAs (miR) miR-130, miR-210 and miR-27b. The authors interpreted these findings as being an adaptive response in these patients to reduce oxidative stress (*i.e.*, antioxidant enzymes upregulation).

All these studies, including our own, measured isoprostanes by ELISA. This may represent a problem, since ELISA assays are less accurate than gas chromatography/mass spectrometry (GC/MS) due, mainly, to cross reactivity [34]. There has been a dearth of follow-up studies comparing ELISA *vs.* GC/MS in PAD. Unfortunately, GC/MS methods are expensive, are not always feasible, and are difficult to implement as routine clinical chemistry tests. Indeed, and apart from any considerations on the precise nature of the molecules measured by the ELISA method, our results showed that the measurement of F_2-isoprostanes (or "F_2-isoprostanes-like substances") by ELISA almost perfectly discriminates between the healthy population and the PAD patients. As such, it is an excellent biomarker of this disease.

Our study confirms the reported increase in plasma CCL2 concentration and decrease in serum PON1 activities and

concentrations in PAD [18,35]. We have previously reported that the ratio between CCL2 and PON1-related variables segregated controls from patients almost perfectly [18]. We showed that the inflammatory response, probably secondary to enhanced oxidative stress, is extreme in PAD patients suggesting that this is causally related to the progression of the disease. A possible explanation for serum PON1 activity being decreased in PAD patients is that PON1 is inactivated by oxidized lipids. This has been demonstrated by studies demonstrating that the incubation of PON1 *in vitro* with oxidized palmitoyl arachidonoyl phosphatidylcholine, lysophosphatidylcholine, and oxidized cholesteryl arachidonate, results in the negation of PON1 activity [15]. We reasoned that patients with PAD represent a clinical model with considerable multiple vessel disease and high burden of atherosclerosis. We found that results obtained from the measurement of plasma CCL2 and serum PON1 concentrations, as well as PON1 paraoxonase and lactonase activities, confirm our hypothesis, and may constitute novel indicators of disease status. In our study, decreased PON1 activities were associated with increased concentrations of CCL2. However, this inverse relationship was not confirmed at tissue level. Indeed, previous studies from our group showed that the expression of both proteins is increased in the arteries of patients with PAD [36]. This observation would suggest that the variations in serum PON1 and plasma CCL2 concentrations do not necessarily correlate with their putative roles at the cellular level. Perhaps PON1 protein expression is increased in diseased arteries to counteract oxidative stress and CCL2-induced inflammation. However, this hypothesis needs to be confirmed by further investigations. An interesting result from our study was to observe an increase in both paraoxonase and lactonase specific activities in PAD patients, due to the decrease in PON1 concentration being higher than that of the enzyme activities. An explanation for this phenomenon cannot be ascertained from the present investigation; albeit we have already observed discrepancies between changes in PON1 activity and its concentration in other non-communicable diseases [37,38]. PON1 activity is strongly dependent on the HDL structure and composition [39,40] and, perhaps, the increased lipid/PON1 protein ratio in the HDL particles of PAD patients increases the enzyme activity as a compensatory mechanism. Currently, however, we can only speculate, and further studies are needed to explore this hypothesis.

In contrast, oxidative modification of proteins does not seem to be important in our patients, since the increase of protein carbonyls is not as marked as that of F_2-isoprostanes. This is consistent with the data contained in previous reports [41,42]. A possible explanation for this relatively small increase in protein carbonyls may lie in the observation that carbonylated proteins have an accelerated turnover since damaged proteins activate proteosomes for their degradation. Thus, carbonylated proteins enter the degradation pathway rather than the chaperone/repair pathway *i.e.* carbonylation represents an irreversible, and non-repairable, modification [43,44].

The results from the present investigation suggest that F_2-isoprostanes and CCL2 are superior to hs-CRP and B2M in the diagnosis of PAD, especially at the earliest stages of the disease. Several studies reported that serum hs-CRP concentrations are elevated in PAD patients, and that the values correlated with ABI and endothelial function [7,45,46]. More recent studies identified B2M protein concentrations as being higher in PAD [47,48]. However, the reported elevations were moderate and, hence, panels of several biomarkers were proposed to enhance their diagnostic accuracy. One of these panels included cystatin C, hs-CRP, B2M, and glucose but, despite this recommendation, the AUROC was no higher than 0.70 [49]. Another study proposed the inclusion of the genetic marker the rs10757269; a polymorphism at the chromosome 9p21 locus. When added to B2M, cystatin C, and hs-CRP, the discriminatory power of the panel was increased considerably. However, the main drawback of this proposal is that genetic markers may be influenced by ethnicity, and this issue has not been sufficiently investigated in the case of rs10757269 polymorphism [50]. Another proposed panel included 9 different biomarkers: hs-CRP, interleukin-6, tumor necrosis factor receptor-II, lipoprotein(a), N-terminal pro-brain natriuretic peptide, pro-atrial natriuretic peptide, C-terminal pro-arginine vasopressin, osteoprotegerin, and fibrinogen [51]. These multiple panels are costly and difficult to implement in standard clinical practice. Conversely, we suggest that the measurement of a single parameter, F_2-isoprostanes or CCl2, does indeed segregate, almost perfectly, PAD patients from the healthy population. A caveat to the present study is that, being based on a Hospital setting, most of the patients studied are already clinically symptomatic. No doubt a useful biomarker would be that which is able to predict asymptomatic PAD. We found that alterations in the circulating levels of F_2-isoprostanes and CCL2 are considerably higher than those observed for the other parameters we had investigated, and appear earlier-on in the evolution of the disease. Thus, these levels may reflect early stages of the disease and, as such, would be invaluable in discriminating disease from non-disease status. We think that population-based studies in primary health centers (PHC) would be worth implementing to fully ascertain the utility of F_2-isoprostanes and CCL2 in the diagnosis of PAD, since the PHC represents the front-line in the discrimination of disease *vs.* non-disease status. Another question that emerges from our study is whether the intake of certain medications influences the concentrations of plasma F_2-isoprostanes. We had not recorded this aspect in the control group and, as such, we cannot be categorical on this issue. However, the finding that F_2-isoprostanes were already elevated in asymptomatic (or nearly asymptomatic) patients, added to the observation that there were no significant difference in F_2-isoprostanes levels segregated with respect to any particular medication, would suggest that the influence of this variable was minimal, if at all.

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Conflict of interest

None.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.07.011.

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Article

Galectin-3 in Peripheral Artery Disease. Relationships with Markers of Oxidative Stress and Inflammation

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Abstract: Galectin-3 is a modulator of oxidative stress, inflammation, and fibrogenesis involved in the pathogenesis of vascular diseases. The present study sought to characterize, in patients with peripheral artery disease (PAD), the localization of galectin-3 in arterial tissue, and to analyze the relationships between the circulating levels of galectin-3 and oxidative stress and inflammation. It also sought to compare the diagnostic accuracy of galectin-3 with that of other biochemical markers of this disease. We analyzed femoral or popliteal arteries from 50 PAD patients, and four control arteries. Plasma from 86 patients was compared with that from 72 control subjects. We observed differences in the expression of galectin-3 in normal arteries, and arteries from patients with PAD, with a displacement of the expression from the adventitia to the media, and the intima. In addition, plasma galectin-3 concentration was increased in PAD patients, and correlated with serologic markers of oxidative stress (F2-isoprostanes), and inflammation [chemokine (C−C motif) ligand 2, C-reactive protein, β-2-microglobulin]. We conclude that the determination of galectin-3 has good diagnostic accuracy in the assessment of PAD and compares well with other analytical parameters currently in use.

Keywords: atherosclerosis; F2-isoprostanes; galectin-3; oxidative stress; peripheral artery disease

1. Introduction

Galectins are a lectin family able to bind to β-galactoside groups [1]. To-date, 14 mammalian galectins have been identified, all of which contain a carbohydrate-recognition-binding domain of 130 amino acids. Galectin-3 is a 29- to 35-kDa protein consisting of two domains, the C-terminal carbohydrate recognition binding domain, and the N-terminal domain that has a unique short end continuing into a Pro-Gly-Ala-Tyr-rich repeat motif [2]. Galectin-3 has been recognized increasingly as a modulator of multiple biological functions such as oxidative stress, proliferation, macrophage chemotaxis, phagocytosis, neutrophil extravasation, neutrophil migration during venous thrombosis,

apoptosis, vacuole lysis after infection, fibrogenesis, and angiogenesis [3–6]. Relationships with oxidative stress have been demonstrated in vitro, such that treatment of monocytes with phorbol myristate acetate, a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent inducer of reactive oxygen species produced an increase in galectin-3 mRNA and protein expression, while blocking with apocynin reversed these effects [7].

Recent evidence suggests that galectin-3 plays a role in the pathogenesis of numerous disease conditions including cancer, as well as inflammatory and metabolic disorders [8–11]. The role of galectin-3 in atherosclerosis deserves special attention. Inactivation of galectin-3 gene or therapeutic modulation of the protein levels was shown to halt the progression of cardiac remodeling, attenuate myocardial fibrogenesis, reduce the atherosclerotic lesion size, and preserve ventricular function in rats and mice [12–15]. Further, galectin-3 is involved in vascular smooth muscle cell osteogenic differentiation [16], myocardial fibrosis and inflammation [17], together with a strong suggestion of association with the risk of cardiac fibrosis, hearth failure, and mortality in the general population, and in patients with atherosclerosis [7,18–20].

In contrast to cardiac disease, information on galectin-3 in peripheral artery disease (PAD) is scarce. Lower-extremity PAD is a frequent manifestation of atherosclerosis that is associated with extensive impairment of different arterial territories. The prevalence of this disease increases with age and in people over the age of 70 years it is estimated to be about 20% [21]. Oxidative stress and inflammation are important factors for the initiation and progression of PAD, and the inflammatory mediators involved in this process are similar to those contributing to coronary artery disease [22,23]. A differential characteristic of PAD is that the associated atherosclerosis affects more extensive regions of arteries and, consequently, the associated biochemical alterations are often more marked than in coronary artery disease [24]. The possibility of finding efficient, non-invasive biomarkers for the early diagnosis of PAD is currently being investigated. Several studies proposed C-reactive protein (CRP) measured by a high-sensitivity method, or β-2-microglobulin (B2M), as useful markers of this disease [25,26]. We recently reported that the measurement of the serum concentrations of F2-isoprostanes and/or the chemokine (C−C motif) ligand 2 (CCL2), markers of oxidative stress and inflammation, may also constitute excellent biomarkers for the diagnosis of PAD [27].

The present study characterized the localization of galectin-3 in arterial tissue, and evaluated the relationships between the circulating levels of galectin-3 versus oxidative stress and inflammation. The diagnostic accuracy of galectin-3 compared favorably with other biochemical markers of these processes in patients with PAD, relative to control individuals.

2. Results

2.1. Histological and Immunohistochemical Analyses

In normal arteries, galectin-3 expression was evenly distributed throughout the adventitia of the artery wall. We observed increased staining in certain areas, coinciding with inflammatory infiltrates. We did not see any specific staining in the tunica media or the tunica intima. We observed a faint positive labeling for CD68 antigen (a marker of macrophages) in the adventitia, but not in the media or the intima, and a positive labeling for α-actin (a marker of smooth muscle cells) in the media (Figure 1).

Arterial samples from patients with PAD had an increased intima thickness with respect to the media (intima/media ratio: 2.06 (0.52–6.19) versus 0.13 (0.10–0.16); $p = 0.002$). Subjects with the less thickened intima layer showed galectin-3 localization mainly together with smooth muscle cells of the media and some positive staining in the remains of inflammatory infiltrates around the vessels of the adventitia, but not in the intima (Figure 2). In contrast, arteries from patients with the more thickened intima showed staining for galectin-3, CD68 and α-actin in the intima (Figure 3).

There were no significant differences in the percentage positive staining for galectin-3 between patients and controls [3.55% (0.60–13.69)% versus 7.11% (0.93–11.04)%, respectively; $p = 0.391$].

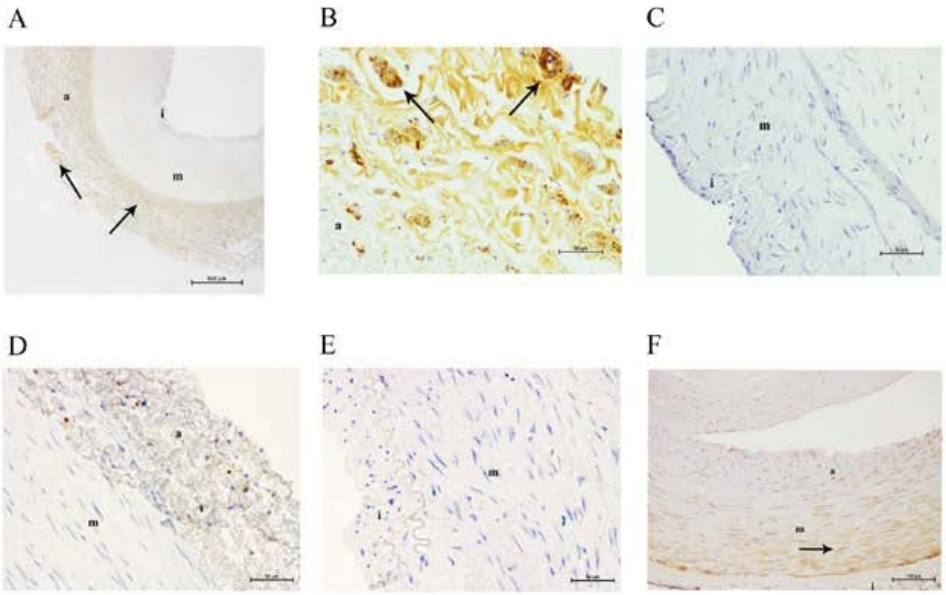


Figure 1. Immunohistochemical images of a normal femoral artery. (**A**) Galectin-3 staining in adventitia at a magnification of 40×; (**B**) galectin-3 staining in the adventitia at a magnification of 200×; (**C**) lack of galectin-3 staining in tunica media at a magnification of 200×; (**D**) faint CD68 staining in the adventitia at 200× magnification; (**E**) lack of CD68 staining in the media at 200× magnification; (**F**) α-actin staining in the media at 100× magnification. **a**, adventitia; **m**, media; **i**, intima. The arrows show positive immunostained areas.

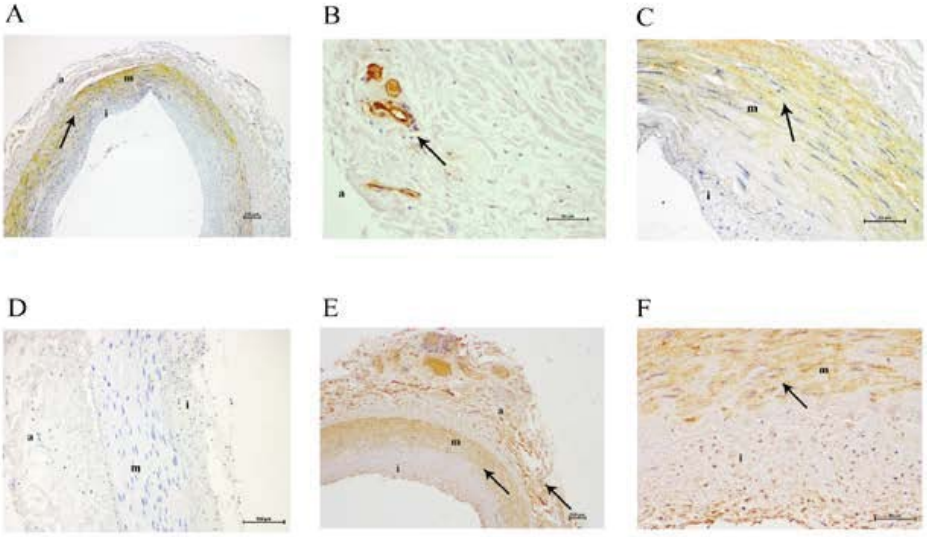


Figure 2. Immunohistochemical images of a moderately altered femoral artery. (**A**) Galectin-3 staining at 40× magnification; (**B**) galectin-3 staining in adventitia at 200× magnification; (**C**) galectin-3 staining in tunica media at 200× magnification; (**D**) lack of CD68 staining at 100× magnification; (**E**) α-actin staining at 40× magnification; (**F**) α-actin staining at 200× magnification. **a**, adventitia; **m**, media; **i**, intima. The arrows show positive immunostained areas.

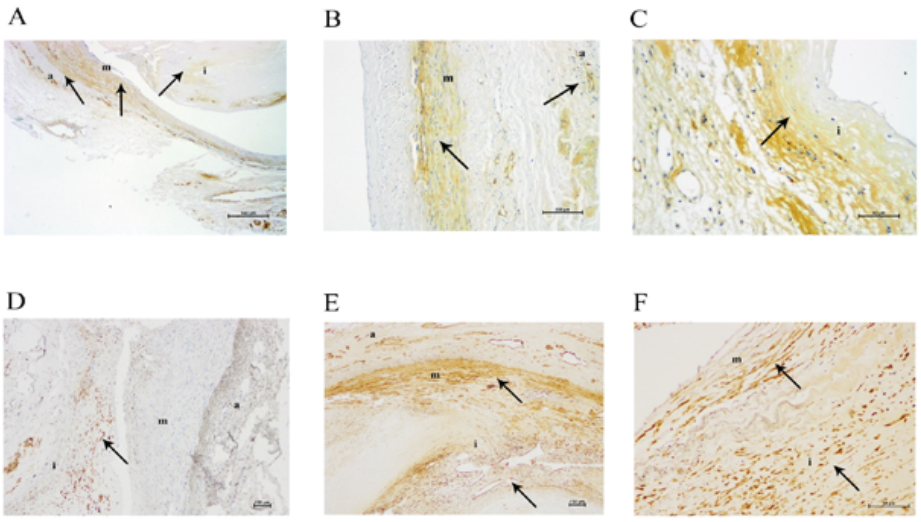


Figure 3. Immunohistochemical images of a severely altered femoral artery. (**A**) Galectin-3 staining at 40× magnification; (**B**) galectin-3 staining in adventitia at 100× magnification; (**C**) galectin-3 staining in tunica intima at 200× magnification; (**D**) CD68 staining at 100×magnification; (**E**) α-actin staining in adventitia, media, and intima at 100× magnification; (**F**) α-actin staining in media and intima at 200× magnification. **a**, adventitia; **m**, media; **i**, intima. The arrows show positive immunostained areas.

2.2. *Biochemical Analyses*

Fifty-three percent of patients were at stages III and IV of the Fontaine classification, and 46% were at stages I and II. There were no significant differences between patients and controls with respect to age and smoking habit. PAD patients were more frequently male, and more suffered from arterial hypertension, diabetes mellitus, dyslipidemia, ischemic heart disease, and chronic obstructive pulmonary disease. There were significant increases in plasma galectin-3, F2-isoprostanes, CCL2, B2M, and CRP concentrations in PAD patients, compared with the control group (Table 1). Plasma galectin-3 and age were not significantly correlated ($r = 0.142$; $p = 0.076$). There were no significant differences in plasma galectin-3 concentrations when patients were segregated according to the other clinical and demographic variables (Table 2). We found significant direct correlations between plasma galectin-3 and F2-isoprostanes, CCL2, CRP, and B2M concentrations (Table 3).

We subsequently segregated PAD patients according to mild disease (Fontaine Stages I and II), or severe disease (Fontaine Stages III and IV); we did not observe any significant differences either in plasma galectin-3 concentrations, or in any of the other biochemical variables studied; the concentrations were increased with respect to the control group (Figure 4).

The diagnostic accuracy of all the selected variables in discriminating between the healthy volunteers, and the patients with PAD, was high. However, plasma galectin-3 measurement was not more efficient than the classical CRP and B2M markers. The order of the calculated accuracies was: F2-isoprostanes ≈ CCL2 > CRP ≈ B2M ≈ galectin-3 (Figure 5). The ratios between galectin-3 and B2M, or CRP significantly improved the diagnostic accuracy of galectin-3 alone (Figure 6).

Table 1. Demographic, clinical, and biochemical characteristics of the control group and peripheral artery disease (PAD) patients.

Variable	Control Group (n = 72)	PAD (n = 86)	p-Value
Age, years	63 (59–73)	66 (49–87)	0.529
Male gender, n (%)	47 (65.3)	68 (79.1)	0.039
Smoking, n (%)	17 (23.6)	6 (10.9)	0.048
Medications, n (%)			
Antiplatelet drugs	Not recorded	25 (29.0)	
Statins		48 (55.8)	
Antidiabetic drugs		48 (55.8)	
Angiotensin converting enzyme inhibitors		48 (55.8)	
Angiotensin receptor antagonists		41 (47.6)	
Calcium receptor antagonists		55 (63.9)	
Diuretics		46 (53.5)	
Anti-arrhythmia drugs		73 (84.9)	
Beta-blockers		73 (84.9)	
Bronchodilators		59 (68.6)	
Arterial hypertension, n (%)	12 (16.9)	28 (63.6)	<0.001
Diabetes mellitus, n (%)	4 (5.6)	29 (67.4)	<0.001
Dyslipidemia, n (%)	7 (9.9)	20 (46.5)	<0.001
Ischemic heart disease, n (%)	0	4 (22.2)	
Chronic obstructive pulmonary disease, n (%)	0	9 (25.0)	
Ankle brachial index = 0.4–0.9	Not applicable	81 (94.2)	
Ankle brachial index < 0.4	Not applicable	5 (5.8)	
Fontaine classification			
Stage I, n (%)	Not applicable	3 (3.4)	
Stage II, n (%)	Not applicable	37 (43.0)	
Stage III, n (%)	Not applicable	9 (10.5)	
Stage IV, n (%)	Not applicable	37 (43.0)	
Galectin-3, ng/mL	6.13 (3.05–12.2)	10.79 (4.21–19.09)	<0.001
F2-isoprostanes, pg/mL	7.76 (3.03–14.82)	90.91 (47.62–141.71)	<0.001
Chemokine (C–C motif) ligand 2, pg/mL	136.34 (88.37–203.22)	565.75 (211.00–1154.00)	<0.001
β-2-microglobulin, mg/L	1.53 (1.09–2.35)	2.22 (1.34–4.55)	<0.001
C-reactive protein, mg/L	0.19 (0.02–0.74)	0.80 (0.07–2.82)	<0.001

Table 2. Plasma galectin-3 concentrations (ng/mL) in PAD patients segregated according to the presence or absence of the selected clinical variables.

Variable	No	Yes	p-Value
Male gender	11.22 (5.50–19.13)	10.60 (4.07–19.98)	0.361
Smoking	10.84 (4.57–19.00)	10.53 (18.89–26.55	0.948
Arterial hypertension	8.79 (3.29–16.76)	10.64 (5.13–23.12)	0.222
Diabetes mellitus	10.57 (3.29–24.69)	10.18 (4.08–19.00)	0.726
Dyslipidemia	10.10 (3.46–20.61)	10.20 (4.11–24.41)	0.932
Ischemic heart disease	8.79 (6.55–18.31)	9.43 (4.88–24.69)	0.878
Chronic obstructive pulmonary disease	9.72 (4.06–22.14)	11.45 (3.29–21.19)	0.349

Table 3. Correlations between plasma galectin-3 concentrations and the other selected biochemical variables.

Parameter	Spearman's ρ	p-Value
F2-isoprostanes	0.437	<0.001
Chemokine (C−C motif) ligand	0.295	0.005
C-reactive protein	0.341	<0.001
β-2-microglobulin	0.544	<0.001

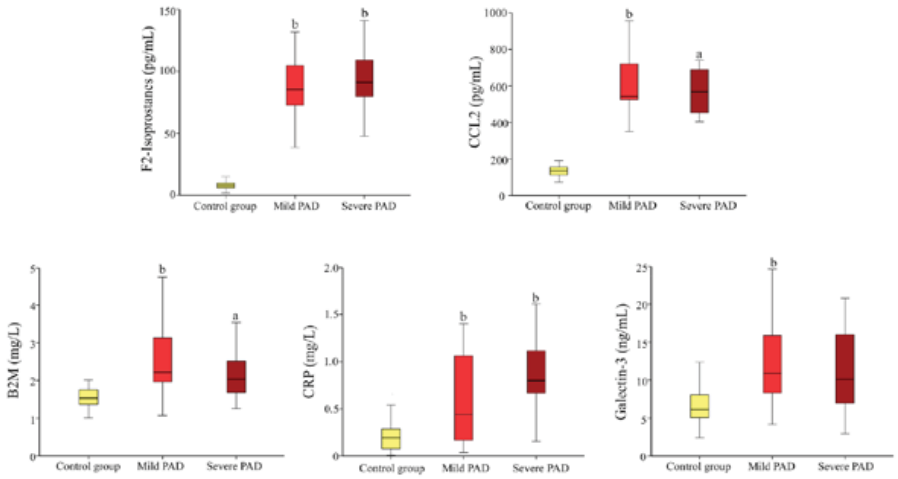
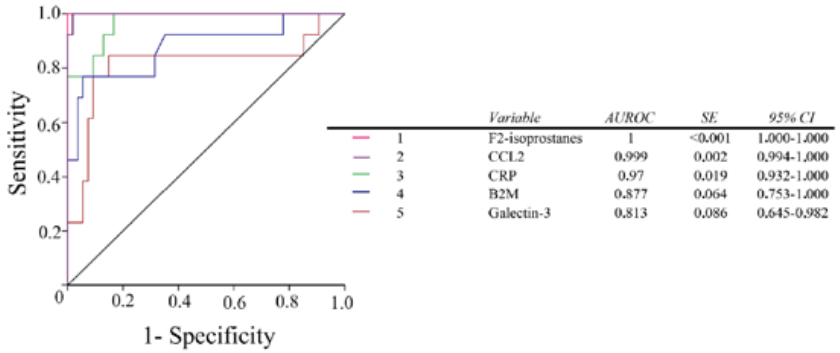
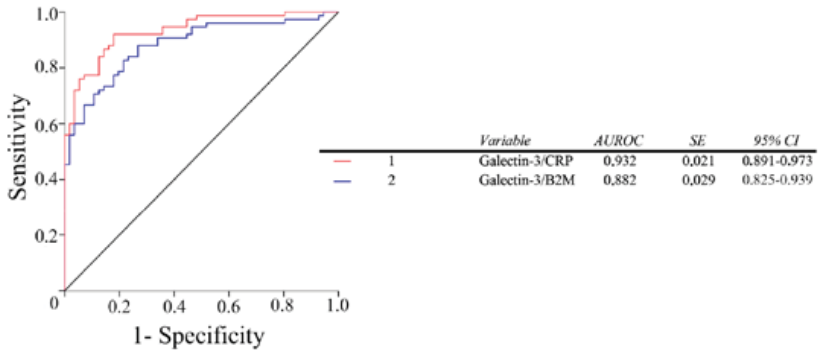


Figure 4. Selected biochemical variables in PAD patients classified according to whether they had mild disease (Fontaine Stages I and II) severe disease (Fontaine Stages III and IV), or were in the control group. Significance values by the Mann–Whitney *U* test: [a] $p < 0.01$; [b] $p < 0.001$, with respect to the control group.



	Variable	AUROC	SE	95% CI
1	F2-isoprostanes	1	<0.001	1.000-1.000
2	CCL2	0.999	0.002	0.994-1.000
3	CRP	0.97	0.019	0.932-1.000
4	B2M	0.877	0.064	0.753-1.000
5	Galectin-3	0.813	0.086	0.645-0.982

Figure 5. Receiver operating characteristics (ROC) plots for all the studied biomarkers in PAD patients and in the control group. AUROC: areas under the curve of the ROC plots. SE: Standard Error.



	Variable	AUROC	SE	95% CI
1	Galectin-3/CRP	0.932	0.021	0.891-0.973
2	Galectin-3/B2M	0.882	0.029	0.825-0.939

Figure 6. Receiver operating characteristics (ROC) plots for the galectin-3/CRP and galectin-3/B2M ratios in PAD patients and in the control group. AUROC: areas under the curve of the ROC plots. SE: Standard Error.

3. Discussion

Galectins, particularly galectin-3, have been gaining importance recently as significant components within the cascade of events underlying the inflammatory reaction and fibrogenesis caused by oxidative stress [2]. Distributions in normal and diseased human peripheral arterial tissue have yet to be defined. Galectin-3 is known to be expressed in a variety of organs in the mouse including the kidney, lung, spleen, colon, uterus, and ovary [28]. In apolipoprotein E-deficient mice (a model of experimental atherosclerosis) galectin-3 is expressed in macrophage-rich areas, but not in smooth muscle cell-rich areas of aortic roots and brachiocephalic arteries [13]. Results of the present study show important differences in galectin-3 distribution in normal arteries, and arteries from PAD patients. Normal arteries showed galectin-3 expression exclusively in the adventitia, while arteries from patients showed the localization mainly adjacent to the smooth muscle cells of the media, with some minor staining in the adventitia and the intima. The pathophysiological interpretation of this finding cannot be inferred from the present investigation, but previous data suggest that this differential tissue distribution could be related to the processes of inflammation, fibrogenesis, and calcification of the atherosclerotic plaque. Indeed, patients with an advanced PAD and atheroma plaque expressed galectin-3 together with macrophages in the intima, suggesting a coordinated role in the formation of the plaque. This hypothesis is supported by data demonstrating that galectin-3 stimulates myofibroblast activation and fibrosis in several types of cells [29,30]. In aortic valves from patients with aortic stenosis, galectin-3 co-localized with the α-smooth muscle cell markers actin and vimentin. It also localized with osteogenic markers such as osteopontin, bone morphogenetic protein 2, runt-related transcription factor 2, and sex-determining region Y-box 9 [31]. Reports indicate that human carotid plaques express galectin-3 in smooth muscle cells, especially in macrocalcified areas, where it co-localizes with alkaline phosphatase [16]. Of note from an early study from our group is the observation of calcium deposition in the media of arteries from PAD patients; a distinct and frequent finding in affected arteries [32]. In the same study, we reported an increase in CD68-positive cells in diseased arteries; the conclusion drawn is that, under certain stimuli, arterial smooth muscle cells can shift their phenotypes to a macrophage-cell state [33]. In addition, another study from our group showed a similar distribution of CCL2 and its receptors in the intima of PAD patients with advanced disease and atheroma plaque [34]. In combination, these data strongly suggest that the changes in galectin-3 distribution in the arteries of patients with PAD are a component of the progression of, and molecular mechanisms underlying, the plaque formation. A caveat of the present study is that we cannot guarantee that the storage of the samples has not in any way altered the histological structure of the control, or diseased arteries. This possibility is unlikely, but it cannot be completely ruled out.

In contrast to the above, in performing quantitative analyses of galectin-3 expression, we found no significant differences between normal and PAD arteries. This could have resulted from the great difficulty in obtaining normal arteries. We used samples derived from tissue donors who were victims of a traffic accident. Selection for the present study followed histological analysis to exclude the presence of atherosclerosis. This resulted in lowering of the number of samples available and made the statistical analysis less than reliable. However, the confidence interval of galectin-3 staining measurements in patients with PAD covered the values observed in normal arteries. The postulation is that quantitative differences in expression between groups, if any, are not of high importance.

Alterations in plasma galectin-3 concentrations in PAD have been investigated by two independent research groups. Casanegra et al. [35] analyzed galectin-3 in 29 patients attending the Mayo Clinics with no evidence of PAD (normal ankle brachial index; ABI), and 31 patients with PAD (low ABI). They observed a mean galectin-3 concentration of 14.4 ng/mL in subjects with normal ABI, and approximately 22% increase in PAD patients. Our results are in the same range of measurement, albeit with some slight differences. Our values in the control group are lower, and the percentage increase in the patient group is approximately 76%. Demographic variation may account for these differences. In addition, the presence of concomitant diseases that could increase galectin-3 levels cannot be ruled-out in the control subjects of the Mayo Clinics' study. Moreover, the PAD populations

studied were different in that we had a higher frequency of patients with resting pain, ulceration, or gangrene. Madrigal-Matute et al. [7] reported plasma galectin-3 concentrations ranging between 2 and 20 ng/mL in PAD patients, with no significant difference with respect to the severity of the disease. This finding is concordant with our observation that plasma galectin-3 concentrations are similar in patients with moderate or severe PAD. They also observed that concentrations above the median were significantly associated with an increase in total mortality risk. A finding of note from our present study is the lack of association between plasma galectin-3 concentrations, and the presence of derangements such as arterial hypertension, diabetes, dyslipidemia, ischemic heart disease, or chronic obstructive pulmonary disease. A possible explanation could be that the interaction of PAD and galectin-3 is stronger than in these other diseases and, as such, masking the influence on the circulating levels of galectin-3. In addition, our patients had been receiving protracted treatment with numerous medications, which can also influence plasma galectin-3 concentrations in these concomitant diseases.

We observed direct correlations between plasma galectin-3 levels and markers of oxidative stress and inflammation, such as F2-isoprostanes, CCL2, CRP and B2M. Galectin-3 was shown [36] to induce oxidative stress through the release of O_2^- in cultured mast cells, an effect that was blocked by the antioxidant enzyme superoxide dismutase. Galectin-3 is also expressed in human monocytes and released under NADPH oxidase-dependent superoxide synthesis [7]. In Wistar rats fed with a high-fat diet [37], leptin increased O_2^- production by a mechanism that requires galectin-3. A recent prospective study in patients with chronic heart failure [38] showed direct correlations between galectin-3, markers of oxidative stress (oxidized low-density lipoproteins and extracellular superoxide dismutase), and markers of inflammation (CRP, interleukin-6), or heart failure (N-terminal pro b-type natriuretic peptide). Those findings are similar to these in the present investigation.

Several studies [18,35] have suggested that the measurement of plasma galectin-3 concentrations may be a good biomarker of diseases related to atherosclerosis. The results from the present investigation show that, in PAD, the diagnostic accuracy of this parameter, albeit quite high, is not superior to that of CRP and B2M, and lower to that of F2-isoprostanes and CCL2. The efficacy of F2-isoprostanes and CCL2 in discriminating between healthy individuals and PAD patients has already been reported by our group [27], and there are no other biochemical parameters identified to date that are superior in diagnostic accuracy. However, the ratios of galectin-3/CRP and galectin-3/B2M also showed a very high efficacy. Further, these measurements have the advantage over F2-isoprostanes, and CCL2, in that they can be easily automated and implemented in routine Clinical Chemistry laboratories, at a low cost and a speed of analysis [39–41]. Studies in wider series of patients need to be conducted to confirm the clinical usefulness of these ratios as PAD biomarkers.

4. Materials and Methods

4.1. Ethics Approval

The Hospital's Ethics Committee (Institutional Review Board) approved the procedures of the study (approval documents 10-04-29/4proj3 and 2011-10-27/10proj1), and written informed consent was obtained from all participants.

4.2. Clinical Assessment of PAD Severity

The extent of PAD was determined using the Fontaine classification, which defines four stages: Stage I, asymptomatic; Stage II, intermittent claudication; Stage III, rest pain; Stage IV, ulceration or gangrene [42]. We also employed the ankle-brachial index (ABI), defined as the ratio of the systolic blood pressure at the ankle to that in the upper arm. Compared to the arm, lower blood pressure in the leg is an indication of blocked arteries due to PAD [43].

4.3. Participants

4.3.1. Arteries from Normal Subjects and PAD Patients

We analyzed portions of femoral or popliteal arteries from patients obtained during surgical procedures for infra-inguinal limb revascularization in the Vascular Surgery Department of Hospital Universitari Joan XXIII between January 2014 and June 2016 (n = 50). All samples were from patients at Stages III and IV of the Fontaine classification. Four normal arteries obtained from accident victims between March 2014 and August 2015, and stored at the Blood and Tissue Bank of Catalonia (Banc de Sang i Teixits, www.bancsang.net/es/donants/donacio_teixits.html, Barcelona, Spain) were used as controls. All samples were stored at −80 °C until processed for histological examination.

4.3.2. Characteristics of Subjects for the Biochemical Study

We underwent an observational, prospective, cross-sectional study in patients attending the Vascular Surgery Department of Hospital Universitari Joan XXIII. Diagnosis of PAD was performed by measuring the ABI, together with non-invasive imaging and angiography, when indicated. We recruited 86 patients with symptomatic PAD (79.1% men, 42–89 years old), 95% having an ABI between 0.4 and 0.9, and 5% with an ABI lower than 0.4. Degree of PAD was determined using the Fontaine classification; 2.6% of the patients were classified as Stage I; 43.6% as Stage II; 10.3% as Stage III; and 43.6% as Stage IV. Patients with clinical or analytical evidence of infection, acute ischemia, renal failure, liver disease, cancer, or autoimmune diseases were excluded. Diabetes, hypertension and dyslipidemia were defined according to established criteria.

The control group was composed of 72 plasma samples obtained from healthy volunteers participating in a population-based study (65.3% men, 58–79 years old). Participants in this study were randomly drawn from the local government census of three communities in the Mediterranean region of Tarragona (Northeast Spain). All members of the control group underwent a physical examination and a blood test. They could walk without any problems or pain and were ostensibly healthy with no clinical or analytical evidence of infectious disease, renal insufficiency, hepatic damage, neoplasia, oligophrenia, or dementia. Their serum concentrations of CRP and B2M were within the normal range. Medication intake was not an exclusion criterion, except in the case of drugs interfering with vitamin metabolism (methotrexate, tuberculostatics, theophylline, or vitamin B6 antagonists). The population studied did not consume vitamin supplements, or local food fortified with vitamins. Pregnant and recent post-partum women were not included in the study. All plasma and serum samples were collected between 2014 and 2016, and stored at −80 °C in our Biological Sample Bank.

4.4. Histological and Immunohistochemical Study

Arteries were rinsed in phosphate buffer to remove residual blood, and placed in at least 10 volumes of buffered formalin using a standard protocol for embedding tissue in paraffin wax for subsequent histology slide preparation. Three sections per slide were used for histological and immunohistochemical analyses. Sections, of 4-µm thickness, were stained with hematoxylin-eosin for histology. The intima and media thicknesses were measured in all histological sections as an estimate of the extent of atherosclerosis. The immunohistochemical expression of galectin-3 was analyzed using goat antibodies against human galectin-3 (dilution of 1/400) from R&D Systems, Inc. (Minneapolis, MN, USA). The appropriate biotinylated secondary antibodies, (Vector Laboratories Inc., Burlingame, CA, USA) were used at a dilution of 1:200. Detection was performed with the ABC peroxidase system (Vector Laboratories, Burlingame, CA, USA), and 3,3′-diaminobenzidine (DAB) peroxidase substrate (Dako Agilent Technologies, Glostrup, Denmark). All immunohistochemical sections were counterstained with Mayer's hematoxylin. The positively-stained areas were quantified automatically (AnalySIS image software system), using an image analysis software (Soft Imaging System, Munster, Germany), and expressed as percentages of the total area. Control tissue samples were processed identically to the test samples, except that the primary antibodies were omitted from the

incubation. The immunohistochemical expressions of CD68 antigen and α-actin were used as markers of macrophages and smooth muscle cells, respectively, and analyzed as previously reported [32,44].

4.5. Biochemical Assessments

Concentrations of galectin-3 were measured in the ethylene diamine tetraacetate (EDTA)-plasma using enzyme immunoassay (R&D Systems®, Minneapolis, MN, USA); Serum F2-isoprostanes and CCL2 were determined by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI, USA, and Prepotech, London, UK, respectively). Serum high-sensitivity CRP and B2M concentrations were measured by automated immunoturbidimetry (Roche Diagnostics, Mannheim, Germany) in a Roche Modular Analytics P800 system (Roche Diagnostics, Basel, Switzerland).

4.6. Statistical Analyses

Differences between any two groups were assessed with the χ^2 test (categorical) or the Mann–Whitney *U* test (continuous), since most of the variables studied had non-parametric distributions. The Spearman correlation coefficient was used to evaluate the degree of association between variables. The diagnostic accuracy of the measured biochemical variables was assessed by ROC curves. This analysis represents plots of all the sensitivity/specificity pairs resulting from varying decision thresholds. Sensitivity (or true positive rate) is the proportion of the sample correctly identified as being specific to the disease. Specificity (or true negative rate) is the proportion of subjects correctly identified as not being specific to the disease. The false positive rate was calculated as 1-specificity. The area under the curve (AUROC) and 95% confidence interval (CI) were calculated. The AUROC represents the ability of the test to correctly classify patients, with respect to the investigated parameter alteration. The values of AUROC can range between 1 ("perfect" test) and 0.5 ("worthless" test) [45].

5. Conclusions

We observed differences in the expression of galectin-3 in normal arteries, and arteries of patients with PAD, with a displacement of the expression from the adventitia to the media and the intima, which may suggest involvement of galectin-3 in the site of the atherosclerosis plaque formation. In addition, plasma galectin-3 concentration was increased in PAD patients, and correlated with serologic markers of oxidative stress and inflammation. The measurement of galectin-3 had a similar diagnostic accuracy to that of CRP and B2M, in the diagnosis of PAD. However, ratios of galectin-3/CRP and galectin-3/B2M showed improved usefulness as biomarkers in PAD.

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Author Contributions: Anna Hernández-Aguilera and Vicente Martín-Paredero were responsible for patient management and clinical data collection; Isabel Fort-Gallifa, and Anna Hernández-Aguilera took responsibility for the biological samples and the database; Isabel Fort-Gallifa, Anna Hernández-Aguilera, Anabel García-Heredia, Noemí Cabré, Fedra Luciano-Mateo and Josep M. Simó performed the biochemical and histological analyses; Isabel Fort-Gallifa, Jorge Joven and Jordi Camps performed the statistical analyses and interpreted the results; Jordi Camps wrote the manuscript, which was subsequently discussed and modified by the rest of the team. All the authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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UNIVERSITAT ROVIRA I VIRGILI
BIOMARCADORES RELACIONADOS CON EL ESTRÉS OXIDATIVO EN LA ENFERMEDAD ARTERIAL PERIFÉRICA
Isabel Fort Gallifa

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New Trends in Biomarkers and Diseases: an Overview

Chapter 12: Emerging serum markers in Peripheral Artery Disease.

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CHAPTER 12

Emerging biomarkers in peripheral artery disease

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Abstract: Peripheral artery disease is, probably, the most common cardiovascular disease. It affects extensive areas of arteries in the lower extremities. Almost invariably it is the result of a sustained and asymptomatic progression of the disease, and effective preventive measures are applied too late. In peripheral artery disease, the degree of atherosclerosis is greater than in patients with adverse events in other arterial territories. Different aspects of atherosclerosis may be represented, and designing strategies for prompt identification could facilitate therapeutic goals. The main objective of this chapter is to describe biomarkers that can be measured in serum or plasma, which help diagnose, monitor and/or predict the course of the disease. We define and describe established biomarkers, emerging biomarkers and candidate biomarkers. We include short descriptions of their main characteristics and their usefulness in the clinical evaluation of peripheral artery disease. Our aim is to give the reader a perspective of the advances made in the research laboratory over recent years in the study of these patients.

Keywords: Atherosclerosis; biomarkers; inflammation; oxidative stress; peripheral artery disease, C-reactive protein, β-2-microglobulin, isoprostanes, PON1, CCL2, protein carbonyls, homocysteine, β-hydroxybutyrate, tricarboxylic acid cycle products, arginine, cysteine, hydroxycholesterol, galectin-3, melatonin, 8-hydroxy-2-deoxy guanosine.

INTRODUCTION

Peripheral artery disease: Clinical and epidemiological characteristics

Lower extremity peripheral artery disease (PAD) is a major health concern since it could result in catastrophic events in different arterial territories such as the extracranial carotid, upper extremity arteries, mesenteric and renal circulation, and arteries of the lower extremities [1].

The incidence of this disease increases with age and, in patients >55 years old, the prevalence of PAD in the legs is approximately 20% [2, 3]. The burden of atherosclerosis is much higher than in patients with adverse events in other arterial territories, and preventive measures are only effective in the initial stages of the disease.

Unfortunately, the initial stages of PAD are often asymptomatic, and appropriate preventive measures are applied too late. In 2010, this disorder affected between 8 and 12 million people in the USA; an increasing prevalence in Europe and Asia is being expected in the near future [4]. A recent meta-analysis estimated that 202 million people worldwide are affected with the disease; the analysis indicating an increase in PAD prevalence of 23.5% in the first decade of the 21st century [5].

Total or partial obstruction of lower extremity arteries is caused by poor circulation resulting in intermittent claudication, characterised by pain in leg muscles during walking; the pain being relieved within a few minutes of stopping. It is the initial symptom of the disease, and should not be confused with pain caused by pinched or damaged spinal nerves i.e. a pain which does not disappear when walking is suspended [1]. Progression of the illness causes pain even at rest, and it is accentuated when legs are raised. The pain can appear in calf muscles, or feet. The most severe stages of PAD are associated with tissue hypo-perfusion resulting in difficulty in wound healing that can progress to ischaemia, ulceration and gangrene; in one third of patients this situation can lead to amputation of the limb [1]. Presence of pain at rest or tissue loss, known as critical limb ischaemia, is associated with about 20% mortality/year.

Clinical evaluation of PAD needs to consider different aspects of the disease: history, epidemiology, and risk factors [6] which are factors predictive of illness progression if not addressed. Risk factors of atherosclerosis in PAD are similar to those in coronary artery disease (CAD). The most important risk factors are: race (being Black is associated with twice the risk for developing PAD compared to White race); age (older members of the population have a higher risk of suffering this disease than do younger members); sex (being male carries a higher risk than being female) [7]. Other risk factors of premature PAD include smoking, diabetes, hypertension, higher circulating concentrations of cholesterol, triglycerides, and homocysteine. High blood fibrinogen concentration is a thrombosis risk factor, and an elevated haematocrit is identified as a factor promoting hyper-coagulation [8]. High-density lipoprotein cholesterol to total cholesterol ratio is, currently, the best biochemical predictor of PAD, according to the consensus document (TASMCII) for the management of PAD [6].

Epidemiological studies indicate that the prevalence of PAD is between 3 and 10% in the general population, but this range increases up to 20% if 70 years of age is used as the cut-off point. These results from the presence of different comorbidities associated with age; essentially increased oxidative stress [1, 6]. The ankle-brachial index (ABI) has been, and still is, used as a screening method for assessing the severity of PAD. ABI compares systolic blood pressure in the upper limbs *versus* that in the lower limbs. Using Doppler ultrasound and a sphygmomanometer, the blood pressure is measured in the arm and the corresponding leg. The index so obtained reflects the extent of PAD. ABI is an effective and objective classification; reduced values of ABI confirm the diagnosis and detect the presence of PAD in asymptomatic patients. It also identifies patients with impaired limb function (difficulty to walk a defined distance, or at a standard walking speed). Finally, it provides important information on prognosis; an ABI 0.9 is associated with a 3-6 fold increased risk of death from cardiovascular disease [9, 10] (Table 1).

Table 1. ABI classification.

ABI value	**Interpretation**
>1.3	Abnormal, non-compressible vessel (calcified)
1.0-1.29	Normal
0.91-0.99	Indeterminate
0.89-0.91	Mild peripheral artery disease
0.41-0.89	Mild-moderate peripheral artery disease
<0.4	Severe peripheral artery disease

However, ABI has a drawback in that diabetic patients, or patients with renal failure, can have non-compressible and calcified arteries in the lower limbs and, therefore, the ABI is falsely elevated. In

these cases the arterial pressure needs to be measured in the toes and related to the measurement in the arm. There are several classifications for PAD, the most widely employed being the Fontaine classification and the Rutherford classification (Table 2).

Table 2. Fontaine and Rutherford classification.

Fontaine classification		**Rutherford classification**		
Stage	Clinical	Grade	Stage	Clinical
I	Asymptomatic	0	0	Asymptomatic
II a	Mild claudication (at distances > 200 meters)	I	1	Mild claudication
II b	Moderate to severe claudication (at distances < 200 meters)	I	2	Moderate claudication
III	Ischaemic pain at rest	I	3	Severe claudication
IV	Ulceration or gangrene	II	4	Ischaemic pain at rest
		III	5	Minor tissue loss
		III	6	Major tissue loss

Arteriography is still the “gold standard” technique, and all classifications need to be compared with it. However, arteriography is a semi-invasive technique and would only be indicated for patients who are candidates for surgery or percutaneous interventions. In addition, the technique cannot be applied to patients with kidney disease because the contrast medium used in the arteriography is contra-indicated in patients with impaired renal function, especially those who have an advanced stage renal disease [11, 12].

Biochemistry and cell biology of atherosclerosis onset and development

Atherosclerosis is initiated by an increase in the inflammation of endothelial cells of the vessel wall associated with low-density lipoprotein (LDL) particles retained within the cells. This phenomenon may be a cause or an effect of the underlying inflammatory process. Atherosclerosis is considered to commence with damage to vascular endothelium caused by a variety of insults such as high blood pressure, tobacco smoking, high cholesterol concentration, diabetes and hyper-homocysteinaemia [13-15].

Plaque formation is initiated by endothelial damage (or dysfunction) in the lumen of the arterial wall. In a second stage, LDL passes through the damaged endothelium and deposits within the artery wall. A hallmark in the development of atherosclerosis is LDL oxidation generating reactive oxygen species (ROS) (Figure 1). ROS are chemical species containing one unpaired electron in the outer orbital of the oxygen molecule. ROS have high biological reactivity and are produced in normal aerobic metabolism in processes such as food metabolism, breathing or physical exercise. ROS production is strictly controlled by the organism with the help of endogenous and exogenous antioxidants. ROS, if uncontrolled, can oxidise and damage essential biomolecules such as polyunsaturated fatty acids (PUFA), DNA, proteins and carbohydrates leading to cellular structure and function damage and, ultimately, cell death [16]. Disequilibrium between ROS production and antioxidant control mechanisms is termed oxidative stress. Direct measurement of ROS is difficult due, in general, to the short half-life of the molecule. Usually, oxidative stress is assessed indirectly as a function of measurable markers.

PUFA present in LDL molecules are particularly sensitive to oxidation. Lipid peroxidation of PUFA has identical general characteristics as that described for oxidative processes in lipids in

cell membranes. Fatty acid peroxidation is a chain reaction consisting of three stages. In the 1st, stage a fatty acid radical is produced when fatty acids react with ROS. The 2nd stage is known as propagation and is due to fatty acid radicals being unstable molecules that react with molecular oxygen leading to a peroxyl radical fatty acid. This is also a highly reactive species that can react with another fatty acid to initiate a chain reaction. The 3rd stage, which only occurs when ROS concentration is high enough, consists of a reaction between two radicals to produce a stable species [17]. This process plays a fundamental role in the onset and development of atherosclerosis [18].

When LDL crosses the damaged endothelium, it is partially oxidised by ROS, and is termed minimally oxidised LDL (MO-LDL). MO-LDL stimulates endothelial cells and smooth muscle cells, which generate the chemokine (C-C motif) ligand 2 (CCL2), formerly termed monocyte chemoattractant protein-1 (MCP1) [19]. ROS also stimulate the expression of adhesines such as intercellular adhesion molecule-1 (ICAM-1 or CD54) and vascular cell adhesion molecule-1 (VCAM-1) and leukocyte adhesion molecules which enhance adhesion of leukocytes to endothelial cells. When monocytes adhere to the endothelial cell surface, MO-LDL is oxidised to OX-LDL. Complete LDL oxidation from MO-LDL is catalysed mainly by myeloperoxidase (an enzyme which is expressed in macrophages found in atherosclerotic lesions) and glycosylases. Some studies have suggested that enzymatic LDL oxidation is possible *in vivo* by interaction with the artery wall, blood cells, plasma constituents and components from the artery wall matrix which are able to hydrolyse cholesterol esters, phosphoglycerides and triglycerides.

PUFA are oxidised by lipo-oxygenases from macrophages, leading to formation of hydro-peroxides from LDL fatty acids. Similarly, plasma cholesterol oxidases are able to generate LDL that is enriched with products from oxidised cholesterol [9, 18]. MO-LDL

induces an inflammatory response leading to apoptosis via activation of nuclear factors, coagulation stimulation, peroxidation induction in lesions, and inhibition of nitric oxide (NO) production. Similarly, OX-LDL produces an inflammatory reaction resulting in lymphocyte T infiltration. Both oxidised molecules, MO-LDL and OX-LDL, are immunogenic and are able to stimulate the release of auto-antibodies while, in addition, platelet aggregation can be altered. OX-LDL and CCL2 promote monocyte migration into the sub-endothelium. Further, OX-LDL is an inducer of pro-inflammatory molecules such as VCAM-1, ICAM-1, selectin P, and CCL2. The inflammatory process depends, mainly, on two adhesion molecules: selectins (implicated in the deposition of monocytes and lymphocytes in the endothelium) and immunoglobulins (responsible for leukocyte adhesion). Currently, however, there is no clear evidence regarding the factors responsible for increasing local expression of adhesion molecules and cytokines, but the more LDL molecules are oxidised the more pro-inflammatory molecules appear to be generated. Differentiation from monocytes to macrophages is due to monocyte colony stimulating factor release from endothelial cells by MO-LDL. Differentiated macrophages develop a receptor to OX-LDL and these molecules are captured by macrophages via phagocytosis to form foam cells. Eventually, tissue necrosis results [18]. OX-LDL exacerbates intra-cellular endothelial damage while, at the time, the lack of NO generates vasoconstriction which regulates vascular tone and inhibits platelet aggregation [20]. NO derives from L-arginine and is released by endothelial cells. NO functions at the transcriptional level by modulating the signalling pathway of nuclear factor kappa B (NF-κB) and by inhibiting the expression of the *VCAM-1* gene in endothelial cells [1]. Macrophages generate many growth factors involved in the formation of collagen, elastic fibres and proteins. Smooth muscle cell proliferation, monocyte migration together with the synthesis of connective tissue and extracellular matrix are major factors in atherosclerosis progression [9].

Despite these disruptive mechanisms, the organism has several systems to counteract the oxidative stress-mediated alterations. Under laminar blood flow conditions, NO synthase is increased, together with NO levels; its anti-inflammatory and vasodilator actions are enhanced. Increasing NO levels is the first defence mechanism against plaque formation; the two essential functions of NO being to relax the vessels and to inhibit platelet aggregation [9, 21]. Under turbulent flow conditions, such as in arterial branches or when vascular endothelium is dysfunctional, there is an inhibition of NO synthesis, increased VCAM-1 expression, and transformation of monocytes into foam cells [1, 22]. It is not only turbulent flow that induces vascular contraction; ROS have a similar effect. Oxygen (O_2) and superoxide anion (H_2O_2) produce contractions in vessels via two possible mechanisms; directly [23], or indirectly mediated by endothelin-1 or by NO breakdown [24-26]. ROS can induce turbulent flow directly by damaging endothelial cells and reducing NO production. O_2 can react with NO to produce more active ROS, such as peroxynitrile (ONO_2^-). ROS can reduce prostacyclin production and decrease the production of vasodilators in the endothelium. The result is an increase in circulating catecholamines causing vasoconstriction of blood vessels and an increase in blood pressure. Another mechanism is mediated by glucose, which increases sympathetic activity and the increase in blood pressure [9, 18]. All these data imply that ROS, via several different mechanisms, can induce hypertension and atherosclerosis.

The second defence mechanism is provided by HDL which penetrates into the sub-endothelium and is instrumental in binding and removal of intra-cellular cholesterol molecules. In atherogenesis, this mechanism fails because the HDL is insufficient to remove the excess LDL entering the cell. Several studies [19, 27] have demonstrated that HDL particles protect LDL from oxidation by means of, at least, two apolipoproteins with enzyme activity: paraoxonase 1 (PON1) and acetylhydrolase platelet activating factor [19, 27, 28]. PON1 is a hydrolase capable of degrading a wide range of different substrates. It circulates in plasma bound to HDL.

The enzyme is able to hydrolyse phospholipids present in oxidised LDL and, as has been suggested, is responsible for many of the observed beneficial effects (e.g. athero-protective) of HDL [18].

The third defence mechanism is provided by platelets, which adhere to damaged endothelium and release growth factors leading to connective tissue formation.

Cholesterol debris and foam cell accumulation in the sub-endothelium is what leads to the core of atherosclerotic plaque. In a more advanced atherosclerotic plaque, accumulation of foam cells in the intimal endothelium layer occurs, and plaque gradually becomes fibrous as smooth muscle cells accumulate within the lesion and produce extra-cellular proteins that form a fibrous matrix. Calcium also accumulates in atherosclerotic plaque. This plaque progresses with protein production by vascular smooth muscle cells.

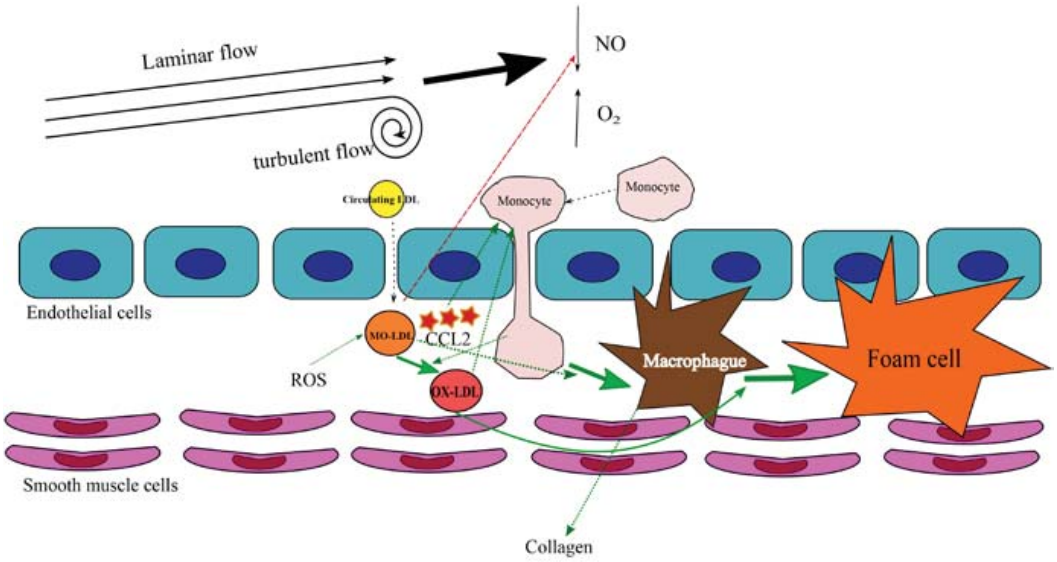

Figure 1: Entry of circulating LDL, MO-LDL transformation, OX-LDL transformation and atherosclerotic plaque formation.

BIOMARKERS

Biomarkers of peripheral artery disease

Definition of "biomarker"

A biomarker is a molecule in the circulation with biological characteristics that can be measured and evaluated; the objective being to improve the physician's ability to assess different aspects of a disease beyond that indicated by the established clinical signs and symptoms. When a biomarker indicates susceptibility to the disease, it is termed a risk factor; when it indicates a capacity to discriminate the affected from the non-affected population, it is a screening biomarker; when it estimates the progression of the disease, it is a staging biomarker; when it estimates the prognosis of the disease, it is a prognosis biomarker; and finally, when it evaluates the efficacy of a treatment, it is a monitoring biomarker.

The accuracy of a biomarker is usually analysed by Receiver Operating Characteristics (ROC) plots. The ROC plot represents the values of 1-specificity for each data point on the x-axis, and the sensitivity on the y-axis. The area-under-the-curve (AUROC) may be between 0.5 and 1.0, and the diagnostic accuracy is higher when this parameter is closer to 1.0 [29, 30].

The search for specific biomarkers for PAD is difficult because this disease and heart disease share risk-factors in common, and have common mechanisms of illness initiation and progression. Comparisons of these diseases indicate similar alterations in: chemokines and inflammatory cytokines; markers of endothelial dysfunction; vascular regeneration mediators; lipoproteins; oxidative stress biomarkers; metabolic modulators and clotting factors. There is no specific biomarker for PAD since all the above are elevated in CAD and other cardiovascular diseases, as well [4]. This is not entirely unexpected but, since the burden of

atherosclerosis is much higher in PAD than in CAD i.e. the extent of the affected arteries is much higher, greater alterations would be expected in patients with PAD, or even specific biomarkers of this condition would be identified.

Established biomarkers

C-reactive protein:

C-reactive protein (CRP) is an acute phase reactant synthesized by the liver. The serum concentrations of this protein increase with inflammation or infection, and also following myocardial infarction, surgical intervention, or trauma [4, 31].

The high-sensitivity method enables the measurement of very low levels of this protein (hs-CRP), and is very useful in establishing presence of inflammation and/or cardiovascular disease (CVD) risk. This analyte is used in combination with other markers such as cholesterol, triglycerides or lipoprotein-a (Lp_a) in determining CVD or PAD risk status. The American Heart Association (AHA) and the Center for Disease Control and Prevention (CDC), defined the following risk groups with respect to CRP [32]:

Low risk: Values <1.0 mg/L
Medium risk: Values between 1.0 and 3.0 mg/L
High risk: Values >3.0 mg/L

β-2-microglobulin:

B-2-microglobulin (B2M) is a low-molecular-weight protein found on the plasma membrane of all nucleated cells. It belongs to the major histocompatibility complex class I. B2M is released into the circulation by nucleated cells, particularly by B lymphocytes and tumour cells. Under normal conditions it is released in very small quantities into the urine; 99% of the filtered B2M being reabsorbed by the renal tubules. High urinary concentrations of B2M are

indicative of renal tubule disease, while increased serum B2M concentrations are found in systemic infection and/or in autoimmune diseases due, in these cases, to increased production rather than decreased clearance. Patients with PAD have elevated serum B2M concentrations due to arterial stiffness, vessel inflammation, decreased renal clearance [11], repeated bouts of ischaemia and reperfusion in the legs [33]. Arterial stiffening reduces the buffering capacity of the elastic arteries which increases systolic and pulse pressure, promotes left ventricular hypertrophy and dysfunction, and impairs the capacity for myocardial perfusion. As such, it plays an important role in atherogenesis and PAD [34].

Emerging biomarkers

This section focuses on describing the results of research into new biomarkers related to oxidative stress and inflammation. Specifically, these molecules such as isoprostanes and carbonylated proteins involve the coordinated roles of PON1 and CCL2 and indices of oxidative stress processes.

Isoprostanes

Isoprostanes are a family of eicosanoids derived from arachidonic acid, and produced by random oxidation. These molecules are generated by oxidation by ROS of polyunsaturated acyl groups *in situ* in membrane phospholipids. Isoprostanes have powerful effects on the vascular system [35]. At least one of the isoprostanes, 8-isoprostane/ 15-F2t-isoprostane/8-iso-15(S)-Prostaglandin $F_{2\alpha}$/ 8-iso-15(S)-Prostaglandin $F_{2\alpha}$/ 8-*iso*$PGF_{2\alpha}$/ $iPF_{2\alpha}$-III/ 8-*epi* $PGF_{2\alpha}$ (Figure 2) is a powerful pulmonary and renal vasoconstrictor, and has been implicated in the pathophysiology of hepato-renal syndrome and pulmonary oxygen toxicity. This molecule has been proposed as a marker of antioxidant deficiency, and oxidative stress [36, 37].

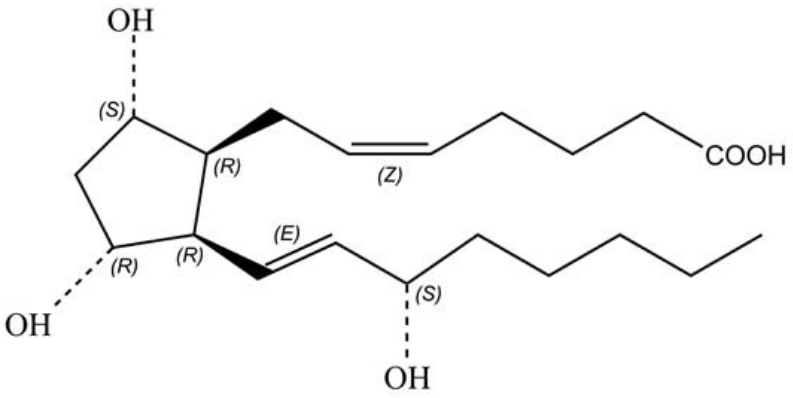

Figure 2: 8-isoprostane (chemdraw.cdx Perkin Elmer software).

Isoprostanes have several modes-of-action: 8-*iso*$PGF_{2\alpha}$ inhibits angiogenesis by blocking vascular endothelial growth factor-induced endothelial cell migration, tube formation and cardiac vessel sprouting. It stimulates tumour growth factor-β that can promote nephropathy associated with type I diabetes. The activity is blocked by antagonists of thromboxane A_2 (TXA_2) receptor, indicating that, in some cases, isoprostanes stimulate TXA_2 biosynthesis.

Plasma from healthy individuals contains small amounts of 8-isoprostane, about 40 to 100 pg/mL. Isoprostanes are considered the most sensitive and reproducible analytes in quantifying lipid peroxidation *in vivo* [38].

These molecules are almost ubiquitously distributed in plasma, cerebrospinal fluid, urine, bile, broncho-alveolar lavage fluid, and in several tissues. The most convenient matrixes for quantification are plasma and urine because they can be obtained without invasive procedures. The values in these fluids are a good reflex of their endogenous production and, thus, provide an accurate index of *in vivo* oxidative stress [39]. Elevation of isoprostanes has been found in several human diseases including atherosclerosis, diabetes, obesity, and pulmonary disease.

There are several methods to quantify isoprostanes in body fluids. These include gas chromatographic/negative-ion chemical ionisation mass spectrometric (GC/MS); liquid chromatographic/mass spectrometric (LC/MS); and immunoassays [40]. One important advantage of LC/MS methods is that the sample preparation is simpler than for GC/MS. Immunoassays are even simpler but have a potential drawback in that there is limited information available regarding precision and accuracy. In addition, there is a paucity of data comparing immunoassays to MS. Despite the limitations of immunoassays, they are successful because of their low cost and ease of methodology. Results obtained by our group have shown that patients with PAD have significantly increased levels of isoprostanes relative to control individuals; AUROC curve analysis of 0.999 demonstrating the high level of accuracy of this measurement in the diagnosis of PAD.

PON1

PON1 belongs to an enzyme family composed of PON1, PON2 and PON3. These three enzymes derive from a common precursor, and their genes are located adjacent to each other on chromosome 7 arm q (21.3-22.1) [41-43].

In humans, PON1 and PON3 expression are observed in most tissues, especially liver, kidney and epithelium. They are found in plasma linked to HDL. PON2 expression is practically ubiquitous in tissues, but it is an intracellular enzyme and cannot be measured in the circulation [13, 44]. PON1 has lactonase, paraoxonase, and arylesterase activities and has been implicated in defence against xenobiotics. PON2 and PON3 have lactonase activity, but not esterase activity. PON1, PON2 and PON3 are able of degrading lipid peroxides in LDL and HDL. PON2 counteracts cellular oxidative stress and apoptosis in endothelial vascular cells.

The enzymes degrade oxidised phospholipids derived from LDL and HDL, as well as from cellular membranes. These features highlight their anti-inflammatory and athero-protective function [19, 45].

In spite the importance of PON1 in the pathophysiology of many diseases, the measurement of this enzyme is difficult to apply in routine clinical chemistry laboratories, due to methodological difficulties. The substrates used for PON1 measurement are not physiological, and some of them are toxic or unstable. These drawbacks preclude automation of PON1 measurement and, as such, it is not included yet in panels of standard biochemical tests in the clinical chemistry laboratory. The most widely used method for the measurement of PON1 activity is the based on the hydrolysis of paraoxon, measured by the increase in absorbance at 412 nm. However, paraoxon has two major drawbacks: it is extremely toxic and very unstable. The solution to the latter problem is to prepare the reagent immediately before use. The solution to the former problem requires that the substrate must be handled in an air-extraction cupboard and the operator has to take safety precautions such as wearing masks and gloves to protect against accidental contact or inhalation of the toxic fumes. The recommended reaction medium is a glycine buffer (0.05 M, pH = 10.5) with 1 mM $CaCl_2$, or a Tris-HCl buffer (0.1 M, pH = 8.5) with 2 mM $CaCl_2$. Some investigators add NaCl (1 or 2 M) to increase PON1 activity. The temperature of reaction can be 37ºC or 25ºC [46-48]. These differences imply that values obtained from different centres vary considerably, and this precludes inter-laboratory comparisons.

Another widely employed substrate is phenylacetate. The reaction buffer is Tris-HCl (9 mM, pH = 8.5) with 0.9 mM $CaCl_2$. The reaction is conducted at 25ºC and monitored at 270 nm [46, 47, 49]. The toxicity or phenylacetate is lower to that of paraoxon, but it also needs to be handled under safety conditions. As we have already described, there is evidence showing that native activity of

PON1 is as a lactonase, and that lipophylic lactones constitute the native substrates. Two tests based on this capacity of PON1 were proposed some years ago [50, 51]. One of the methods uses 5-thiobutyl butyrolactone (TBBL) as a substrate. TBBL is a synthetic lactone that resembles the natural substrate of PON1. The method allows PON1 activity to be analysed using a more 'physiologically-akin' substrate. The lactonase measurement correlates well with the levels of PON1-HDL complex and provides an estimate of the quality of the HDL particles to which the enzyme is attached [51, 52]. The incubation reagent contains 1 mM $CaCl_2$, 0.25 mM TBBL, and 0.5 mM 5,5'-dithio-bis-nitrobenzoic acid in 0.05 mM Tris-HCl buffer (pH = 8.0). The temperature of reaction is 25ºC and the change in absorbance is monitored at 412 nm. The other method employs 7-*O*-diethyl phosphoryl 3-cyano 4-methyl 7-hydroxycoumarin (DEPCyMC) as substrate, and this method is a good estimate of the total PON1 protein mass, whether bound or not to HDL particles. The incubation reagent contains 1 mM $CaCl_2$ and 1 mM DEPCyMC, in 50 mM bis-trispropane (pH = 9.0). The temperature of reaction is 25ºC and the change in absorbance is monitored at 400 nm. These methods have two great advantages over the esterase assays. First, they measure a type of PON1 activity that is closer to the physiology. Second, the TBBL and DEPCyMC substrates are not toxic. During the 1990s, some groups were able to generate monoclonal or polyclonal antibodies against purified PON1 [53, 54]. These antibodies were employed in developing ELISA methods for the measurement of serum PON1 concentration and, as well, in immunohistochemical studies [55-57].

The problem associated with these methods was that the quality of the antibody was very variable, and depended on the quality of the initial purified PON1 preparation. More recently, we developed antibodies against synthetic peptides which are specific of PON1, PON2 and PON3 mature sequences. These peptides are CRNHQSSYQTRLNALREVQ (PON1 specific), CKEEKPRARELRISRGFDL (PON2 specific) and CRVNASQEVEPVEPEN (PON3 specific). These antibodies enabled us to develop immunohistochemical methods to study the

distribution of the three PON in tissues [58], including human arteries [59], and ELISA methods to measure the serum concentrations of PON1 and PON3 [58-61].

Our studies showed that PAD patients have decreased PON1 lactonase and paraoxonase activities, and decreased PON1 concentrations. The best diagnostic accuracy was observed for PON1 concentration, with an AUROC value of >0.95.

CCL2

Chemokines, or chemoattractant cytokines, are a family of small secreted proteins that are globular in structure and which function as messengers to activate and to direct migration of specific subtypes of leukocytes from the bloodstream into injured tissues. Chemokines, in particular CCL2 (chemokine (C-C motif) ligand 2) also termed MCP-1 (monocyte chemoattractant protein 1), play a significant role in the regulation of the inflammatory response [19, 62]. Chemokines are associated with the migration of immune cells and, as well, are of considerable importance in the correct functioning of metabolic pathways [62]. The most-known function of CCL2 is to stimulate monocyte migration to the inflammation sites and, subsequently, to induce their differentiation into macrophages [63]. CCL2 binds to CC chemokine receptor 2 (CCR2), which is expressed on the cell surface, and promotes accumulation of monocytes on the vascular wall [64, 65].

Circulating chemokines cause metabolic perturbations which can be reversed by anti-inflammatory drugs [66]. Both, CCL2 protein and mRNA, are expressed in the majority of tissues, suggesting that there is a systemic production and response to inflammatory stimuli [67]. Thus, CCL2 synthesis is induced in several diseases that feature monocyte-rich cellular infiltrates. These diseases include atherosclerosis, congestive heart failure and rheumatoid arthritis [68]. Many stimuli, particularly oxidative stress, cause the production of CCL2 in vascular cells and stimulation of foam cell

formation, inflammation, and progression of the atherosclerosis process [65, 69]. Increased levels of CCL2 have been observed in PAD, and it has been suggested that CCL2 concentration in plasma can be an index of the severity of the disease. However, this hypothesis needs confirmation [63]. The study of the interrelations between CCL2 and PON1 may provide clues to the understanding of PAD. For example, the increase in the production of CCL2 by endothelial cells may be significantly inhibited by the action of PON1. These results suggest inter-related roles for CCL2 and PON1 molecules [44].

Early studies from our group showed that serum PON1 activity and concentration were significantly lower, and CCL2 concentration higher, in PAD patients compared to control individuals. Our results also showed that the combination of plasma CCL2 and PON1-related variables was capable of almost complete discrimination of control individuals from patients [67, 70, 71].

Protein carbonyls

The most widely used indicator (or marker) of protein oxidation is carbonylated protein content [16]. Carbonylation represents the most frequent oxidative modification of proteins. This modification is chemically stable and is a feature of particular importance in protein storage and in the methodology for the detection of protein carbonyls [16, 17].

Cations generated by the redox cycle, as Fe^{2+} or Cu^{2+}, can bind to cation binding sites of proteins which, when binding to H_2O_2 or O_2, can transform to carbonyls, the amine groups from the protein lateral chain of several amino acids. Protein oxidation catalysed by metals is not the only mechanism by which carbonyls are introduced inside the protein structure. For example, cigarette smoke and aldehydes are also implicated in protein oxidation. Many analytical methods have been developed to detect and quantify the

extent of protein carbonylation in protein preparations. The most used method consists of protein carbonyl derivatisation with 2,4-dinitrophenylhydrazine (DNPH) and subsequent spectrophotometric assay. This method enables an overall quantification of protein carbonyl content due to the ability of DNPH to react with carbonyls to produce an adduct that absorbs light at 366nm. These methods has been sophisticated including a high-performance liquid chromatography to separate molecules and parallel measurement of absorbing adducts. Subsequent, immunological techniques, such as Western blot or ELISA, have been developed leading to an increase in sensitivity of protein carbonyl detection [72].

Currently, these methods are extensively employed in evaluating changes in total protein carbonyls and, eventually, to identify the specific proteins undergoing oxidation. More recently, many mass-spectrometry methods have been developed for the identification of protein carbonyls, and the associated amino acid residues that have been modified to carbonyl derivatives. Although these mass-spectrometry methods are detailed and accurate, due to their ability to identify the amino acid residues undergoing carbonylation, they require expensive equipment, and which limits their general availability [73]. Despite the potential usefulness of this marker in processes in which oxidative stress is implicated, results from our laboratory show that the measurement of protein carbonyls has no greater accuracy than do the measurements of hs-CRP and β2-microglobulin in the diagnosis of PAD.

Homocysteine

Homocysteine is an intermediate metabolite of methionine resulting from the action of the enzyme methionine adenosyl transferase (MAT). It is a sulphur amino acid, the importance of which lies in the transference of methyl groups in cellular metabolism [74]. Methionine is generated from homocysteine re-methylation and enzyme catalysis by homocysteinemethyltransferase (HMT); this enzyme needing vitamin B12 and 5,10-methylenetetrahydrofolate for its function. The 5,10-methylenetetrahydrofolate acts as co-

substrate in the course of its conversion to 5-methylenetetrahydrofolate by the enzyme methylene tetrahydrofolate reductase (MTHFR). Vitamins B6 and B12 act as co-enzymes for methionine and homocysteine metabolism. Hence, there is a direct relationship between these two vitamins and homocysteine levels. Homocysteine contributes to cardiovascular disease through mechanisms that include endothelial dysfunction, increased lipid permeability, and vascular inflammation. The association between hyper-homocysteinaemia and increased oxidative stress has been demonstrated [75]; clinical studies have shown an incidence of hyper-homocysteinaemia of between 28 and 30% in patients with premature PAD. Thus, hyper-homocysteinaemia appears to be an independent risk factor for the development of this disease [76]. Mutations in the genes for MTHFR and cystathionine beta synthase (CBS) enzymes may alter the metabolism of homocysteine, promoting its increased concentration [77]. The *MTHFR* gene has received attention due to the relationship between folic acid intake and cardiovascular diseases. This gene has several polymorphisms; the C677T mutation being one of them. Patients bearing the 677T allele have lower enzyme activity than those patients with the 677C allele. The 677T allele is associated with an *in vitro* thermo-lability and a reduction of 50% in the enzyme's activity [78, 79].

There are three genotypes: 677CC (homozygote wild type), 677CT (heterozygote mutant) and 677TT (homozygote mutant). Subjects with 677TT have higher circulating homocysteine concentrations. Those with the 677TT allele represent about 10% of the population [80]. A second mutation in *MTHFR* in exon 7 is A1298C which results in a mutation that reduces MTHFR enzyme activity to a lesser extent than does C677T. However, compound heterozygous MTHFR A1298C/C677T carriers may develop hyper-homocysteinaemia.

CBS enzymes can alter the metabolism of homocysteine in favour of its increase [78]. Several mutations have been identified in the

CBS gene, and the presence of these mutations in the homozygous form is associated with hyper-homocysteinaemia. The prevalence of these mutations varies among populations and ethnic groups; 844ins68 and T833C mutations being the most commonly reported to occur in the cis position in carriers of the 844ins68 mutation. The 844ins68 mutation in exon 8 causes the insertion of 68 base pairs in position 844, while the T833C mutation occurs as a replacement of a thymine by a cytosine nucleotide in position 833 [77, 78].

Homocysteine was first measured using amino acid analysers, or by radio-immunoassay. However, these techniques have been replaced with more convenient ones that are more rapid, and do not require the radioactivity of radio-immunoassay. More recently, high resolution liquid chromatography, with electrochemical or fluorescent detectors, as well as enzyme-immunoassays, have been developed. Immunoassay has the advantage of easy automation, enabling the measurement of homocysteine in routine clinical chemistry laboratories. Liquid chromatography linked to a mass-mass detector is the reference method, but it is time-consuming and the instrumentation is not readily available.

Collection and conservation is one of the most important features in homocysteine measurement. To pre-empt transfer of homocysteine from red blood cells into plasma, one of the most important features is to centrifuge the blood sample quickly at 4ºC, and separate the plasma from the rest of the constituents. There are no significant differences between serum and plasma samples with respect to homocysteine measurements. Overall, these data suggest that hyper-homocysteinaemia is an independent risk factor for PAD and CAD [81].

Metabolomics and the search for new biomarker candidates

Metabolomics techniques are revolutionising clinical chemistry analyses, particularly in the search for circulating metabolites that are significantly altered in various diseases; their altered concentrations can be used as biomarkers of disease presence and/or disease status. In this section we propose some metabolites which, although not yet established unequivocally in the evaluation of peripheral arterial disease, there is sufficient evidence to warrant further research.

β-hydroxybutyrate

Ketones such as beta-hydroxybutyrate and acetoacetate are produced in states of negative energy catabolism, or in decreased glucose utilisation such as low carbohydrate ketogenic diets, starvation, or high-intensity exercise. Caloric restriction or fasting reduces inflammation as the organism adapts to low glucose intake. Energy metabolism then switches to mitochondrial fatty acid oxidation, ketogenesis and ketolysis [82]. In the liver, β-hydroxybutyrate levels are increased when there is an excess production of acetyl coenzyme A (acetyl CoA). Acetyl-CoA is enzymatically transformed to acetoacetate which, subsequently, is reduced to beta-hydroxybutyrate. Production of acetoacetate increases during carbohydrate deprivation (fasting, digestion perturbations, vomiting), decreased carbohydrate utilisation (glycogen storage diseases, diabetes mellitus, and alkalosis). The increase in acetoacetate may exceed the capacity of tissues and, as acetoacetate accumulates in the blood, a small amount is converted to acetone by decarboxylation. The remaining; and greater, portion of acetoacetate is converted to β-hydroxybutyrate. This compound is indirectly measured in human serum or urine (ketone bodies analysis) as a specific indicator of diabetic ketoacidosis in diabetes mellitus [83]. Ketone body metabolism comprises three pathways which are non-oxidative metabolic fates of ketone bodies: ketogenesis within hepatic mitochondria (the primary source of

circulating ketone bodies); terminal oxidation within mitochondria of extrahepatic tissues via CoA transferase (primary metabolic fate of ketone bodies); and cytoplasmic *de novo* lipogenesis and cholesterol synthesis [82].

β-hydroxybutyrate levels are likely to be altered in PAD, since artery diseases modify fatty acid metabolism causing an accelerated rate of lactate formation from glucose. Moderately ischaemic tissue from oxidation causes a disruption in cell homeostasis resulting in lactate accumulation and decrease in pH and ATP. This situation can be inhibited by metabolites that reduce fatty acid oxidation and increase the combustion of glucose and lactate [84]. In addition, β-hydroxybutyrate inhibits the NLRP3 inflammasome in response to several NLRP3 activators. The NLRP3 inflammasome is an innate immune sensor that becomes activated in response to damage-associated molecular patterns (DAMPs) such as toxins, excess glucose, ATP, amyloids, ceramides, urate and cholesterol crystals. NLRP3 controls the activation of caspase-1 and the release of the pro-inflammatory cytokines IL-18 and IL-1β in macrophages [85, 86]. From a mechanistic point of view, β-hydroxybutyrate inhibits NLRP3 inflammasome without undergoing oxidation in the TCA cycle. It has other roles which implicate Sirt2 and the receptor Gpr109a. Inhibition of NLRP3 did not correlate with the magnitude of histone acetylation in macrophages; the inflammasome-mediated IL-1β and IL-18 production in human monocytes being inhibited. *In vivo*, this molecule attenuates caspase-1 activation and IL-1β secretion in mice. These data suggest that the anti-inflammatory effects of caloric restriction (or ketogenic diets) are mechanistically linked to inhibition of NLRP3 inflammasome via β-hydroxybutyrate. Hence, there is a potential therapeutic use of β-hydroxybutyrate against pro-inflammatory diseases, by acting against NLRP3 inflammasome [87].

Tricarboxylic acid cycle products

LDL uptake by endothelial cells results in the generation of acetyl-CoA [37], which drives flux through the tricarboxylic acid cycle (TCA cycle). Increased acetyl-CoA alleviates the need for carbohydrate-derived precursors, thereby inhibiting glycolytic flux in the TCA cycle, and elevating the 3-carbon intermediates (Figure 3).

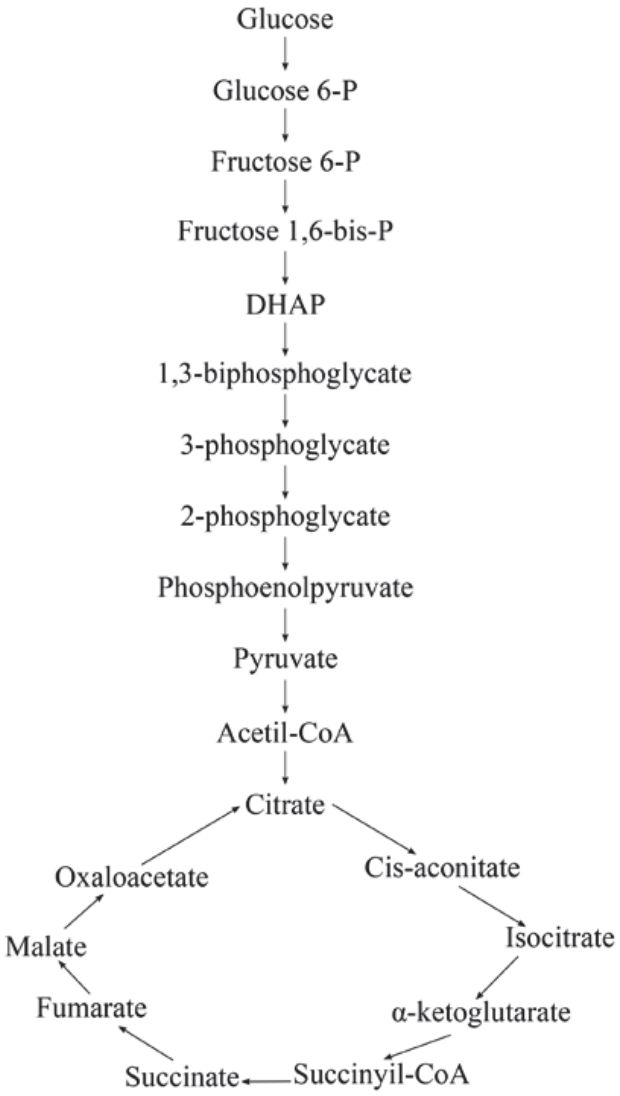

Figure 3: Krebs' cycle (or "citric acid cycle"). Figure adapted from Lehninger. Principios de Bioquímica .

Studies conducted by our group showed that treatment of endothelial cells with oxidised LDL could induce levels of oxidative stress sufficient to impair normal energy pathways. In cells treated with oxidised LDL there was an accumulation of 6-carbon intermediates and a decrease of 3-carbon intermediates. The hypothesis is that this situation may be due to changes in glyceraldehyde-3-phosphate dehydrogenase (GADPH; whether in concentration or activity) in response to oxidised LDL, since superoxide overproduction inhibits GADPH via a mechanism that involves poly-ADP-ribose polymerase (PARP) activation [60]. Following on from this possible explanation, it is necessary to highlight that the TCA cycle intermediates are lowered, probably due to the attenuated conversion of 6-carbon glycolytic intermediates to 3-carbon compounds. This situation feeds into the TCA cycle via two compounds, pyruvate and acetyl-CoA. When normal HDL is added to oxidised LDL-treated cells, there is a partial reversion of energy metabolism pathways.

One could conclude that oxidised LDL promotes the accumulation of 6-carbon glycolytic intermediates, and decreases 3-carbon TCA intermediates due to changes in GADPH via PARP activation. HDL affects this via PON-1. Our group has reported that the addition of normal HDL to oxidised LDL in cells, partially reverses the impact on energy metabolism pathways and, therefore, the concentrations of 3-carbon intermediates, and the TCA cycle, are normalised. However, the addition of HDL from PON1-deficient mice was shown not to normalise these alterations. These results suggest that PON1 has a beneficial role by curtailing the changes in energy metabolism often leading to endothelial dysfunction [27].

Amino acids arginine and cysteine related to TCA cycle

These are glycogenic amino acids which are converted into TCA cycle intermediates, and their concentrations in the circulation have been observed to be increased in PAD. Arginine and its metabolite, homoarginine, increases endothelial NO production; the lack of NO increases vasoconstriction and inhibits platelet aggregation [9].

Several studies have investigated the role of arginine in PAD and its therapeutic supplementation on vascular and metabolic functions. However, results have been inconsistent. Some studies showed that short term complementation of arginine in the diet has positive effects in patients with PAD, but others showed that long-term supplementation could even be harmful. Symmetric dimethyl-arginine (SDMA), a structural analogue of asymmetric dimethyl-arginine (ADMA), inhibits NO formation by inhibiting cellular uptake of L-arginine [9, 88-90]. More studies are needed to define the status and function of arginine and its derivatives in PAD.

Cysteine is a non-essential amino acid, and is usually synthesized from serine and methionine. Cysteine plays a role, as a component of glutathione, in an endogenous defence mechanism to decrease oxidative stress. But cysteine is also a component of CCL2, a cysteine-cysteine protein, and its plasma concentration is decreased in PAD, probably due to increased CCL2 synthesis [91].

Other biochemical candidates

Hydroxycholesterol

The ‘Oxysterol Hypothesis’ was formulated by Kandutsch and his team in 1978. The assertion was that the negative feed-back of cholesterol on its own synthesis is mediated not by cholesterol itself, but by oxygenated forms of cholesterol i.e. the so called oxysterols. Currently, oxysterols are considered end-products of cholesterol metabolism; they are oxygenated sterol derivatives and can be biologically potent. However, the half-life is short [92, 93].

The field of oxysterol research has been revitalised recently since these molecules have been implicated in the development and progression of atherosclerosis. However, there is no consensus regarding their involvement in atherosclerotic lesions. Currently, it is known that 27-hydroxy cholesterol (27HC) is increased in atherosclerotic lesions, but little is known of its role in

atherogenesis. Other potentially interesting hydroxycholesterols are 24-hydroxy cholesterol (24HC) which eliminates excess cholesterol from brain. 25HC in humans is exclusively produced in the brain and is involved in immune function together with 27HC which is a selective modulator; both are precursors of bile acid synthesis [93].

Galectin-3

Galectins were identified after the work by Ashwell and Morrell who characterised, in the 1960s, the asialoglycoprotein receptor. Galectins are a family of lectins that bind β-galactosides. They are highly conserved mammalian lectins that regulate inflammation. Galectins are classified into three distinct groups based on their quaternary structure: prototypical, chimeric, and tandem repeat [94].

Galectin-3 is a carbohydrate-binding protein which participates in a variety of biological process including proliferation, macrophage chemotaxis, phagocytosis, neutrophil extravasation, oxidative stress, apoptosis and angiogenesis. All of these mechanisms are involved in cardiovascular diseases [95]. The role of galectin-3 as a biomarker of heart failure has been demonstrated; it seems that galectin-3 has value in predicting heart failure [96, 97], but there a dearth of physiological evidence of its involvement in atherosclerosis. Galectin-3 has a key role in vascular fibrosis, and is known to be involved in monocyte to macrophage conversion.

The study by Madrigal-Matute et al. [95] showed that galectin-3 was implicated in PAD and had a relevant contribution to patient mortality. The authors observed that galectin-3 levels are a significant, and independent, risk factor associated with overall cardiovascular mortality in patients with PAD. Other studies showed that galectin-3 levels were positively associated with nephropathy, heart failure, and PAD, and that galectin-3 could be used as a marker of systemic inflammation via macrophage activation promotion and monocyte attraction. The action is,

predominantly, pro-inflammatory. Neutrophil-endothelial interactions are promoted and numerous cell types involved in inflammatory and immune responses are activated, leading to fibrosis, but essential for tissue repair. Galectin-3 could be acting as an inflammatory factor in vascular disease, thereby accelerating adverse ventricular remodelling [98].

Melatonin

Melatonin is an endogenous hormone synthesized and secreted mainly by the pineal gland. It was isolated and chemically identified as *N*-acetyl-5-methoxytryptamine in the 1960s. Melatonin derives from tryptophan which, in a first step is transformed into serotonin, and in a second step, in melatonin by two enzymatic reactions. Melatonin is a potent free radical scavenger, with antioxidant properties [99].

Experimental studies show that melatonin acts at multiple levels: direct scavenging of free radicals and singlet oxygen; stimulation of antioxidant enzymes activities; inhibition of pro-oxidant enzymes; reduction of radical formation in neurones by anti-excitatory actions; anti-inflammatory properties; reduction of electron leakage by support of mitochondrial electron flux; prevention of oxidative stress by optimising phasing and amplitudes of metabolic circadian rhythms; formation of redox reactive melatonin metabolites with radical-scavenging properties; and, finally, scavenging of low reactivity free radicals by melatonyl radicals, or mediated by catalysts.

Melatonin stimulates gene expression and activity of several antioxidant enzymes such as glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) [99-102]. These data highlight melatonin as a potentially useful biomarker of atherosclerosis, and a possible therapeutic tool for this disease [103].

8-hydroxy-2-deoxy guanosine

8-hydroxy-2-deoxy guanosine (8-OhdG) is an oxidised derivative of deoxyguanosine. It is one of the major products of DNA oxidation. The measurement of 8-OhdG concentration in serum or urine is a good index of oxidative stress. Endogenous oxidative damage to DNA can be measured as an indicator of aging, or as an indicator of oxidative stress; both being related. The extent of damage can be assessed by measuring the steady-state level of 8-OhdG [104]. 8-OHdG is used in measuring endothelial dysfunction in CAD [105]. However, there are few studies that have investigated the relationship of 8-OhdG with PAD [106].

CONCLUSION

The lack of specificity of currently-used established biomarkers such as CRP and B2M limit their usefulness in the study of patients with PAD. In particular, these molecules are often increased in serum when the disease is already clinically symptomatic, and they are not useful in discriminating between different stages of the disease, or of severity. This problem has encouraged investigators to focus on evaluating new markers that have sufficient sensitivity and specificity to identify asymptomatic PAD patients, alternatively, the patients may be diagnosed already and have had clinical intervention. In such cases, these markers may be used to monitor the course of the disease. Results from our group have shown that emerging biomarkers related to oxidative stress and inflammation may be useful in the early diagnosis of PAD. These biomarkers include 8-isoprostanes, CCL2 and PON1. Investigation of these biomarkers and alterations in energy dynamics and metabolism (“metabolomics”) would provide new analytical tools with potential clinical and therapeutic importance.

List of abbreviations:

ABI: ankle-brachial index
CAD: coronary artery disease
CCL2: chemokine (C-C motif) ligand 2
CVD: cardiovascular disease
HDL: high density lipoproteins
ICAM-1: intercellular adhesion molecule-1
LDL: low density lipoproteins
MCP1: monocyte chemoattractant protein-1
NO: nitric oxide
PAD: peripheral artery disease
PON: paraoxonase
ROS: reactive oxygen species
VCAM-1: vascular cell adhesion molecule-1
8-OHdG: 8-hydroxy-deoxyguanosine
24-HC: 24- hydroxy cholesterol
25-HC: 25- hydroxy cholesterol
27-HC: 27- hydroxy cholesterol

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Conflict of interest:

The authors declare none conflict of interest.

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Presentaciones en Congresos:

VII Congreso Nacional de Laboratorio Clínico 2013

LOS 8-ISOPROSTANOS: UN NUEVO MARCADOR PARA DIAGNÓSTICO Y EVOLUCIÓN DE LA ENFERMEDAD ARTERIAL PERIFÉRICA.

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INTRODUCCIÓN:

La progresión silente de la arteriosclerosis como consecuencia del estrés oxidativo y la inflamación crónica en la enfermedad arterial periférica (EAP), podría controlarse con medidas adecuadas de prevención. Sin embargo, se carece de marcadores bioquímicos para su evaluación y diagnóstico precoz.

La especificidad y sensibilidad de los marcadores instaurados en los laboratorios hospitalarios son limitadas. Estudios previos de nuestro grupo han investigado la utilidad de nuevos marcadores de marcadores de estrés oxidativo e inflamación, como son la proteína quimioatrayente de monocitos (MCP-1) y la enzima antioxidante paraoxonasa-1 (PON1).

OBJETIVO:

Estudiar la sensibilidad y especificidad diagnóstica de la concentración sérica de 8-isoprostanos, un marcador de estrés oxidativo, frente a los diferentes marcadores, establecidos y emergentes, de EAP.

MATERIAL Y MÉTODOS:

Se recogieron 115 pacientes de sexo masculino entre 55 y 80 años, diagnosticados clínicamente de EAP y 400 controles con una distribución similar de edad y sexo. Las concentraciones de PCR, colesterol, insulina, HDL-C y triglicéridos se midieron mediante un autoanalizador Unicel™ DxI 800; la concentración de MCP-1 mediante un ensayo ELISA (Prepotech®EC Ltd); actividad lactonasa de PON1 , mediante la capacidad de hidrólisis de 5-tiobutil butirolactona y actividad paraoxonasa de PON1 mediante la capacidad de hidrólisis del paraoxón; la concentración de PON-1 se determinó mediante un ensayo ELISA con anticuerpos de ratón policlonales contra estructuras concretas de PON-1; la concentración de isoprostanos se determinó mediante un ELISA (Cayman® Chemical). En los pacientes con EAP se midió el ABI (ratio de la tensión arterial sistólica medida en el tobillo y el brazo), teniendo en cuenta que un ABI ≤1 es indicativo de EAP.

RESULTADOS:

Los resultados de la mediana e intervalos de confianza del 95% de los diferentes marcadores de EAP del grupo de controles y pacientes se especifican en la siguiente tabla:

	Controles	PAD	p
Isoprostanos (pg/mL)	8.6 (2.4-21.8)	92.3 (36.9-290.7)	<0.001
Colesterol (mmol/L)	5.2 (3.7-7.0)	3.9 (2.6-5.9)	
HDL-C (mmol/L)	1.46 (0.96-2.20)	1.06 (0.59-1.79)	
Triglicéridos (mmol/L)	1.1 (0.5-2.9)	2.9 (1.7-4.8)	
Insulina (pmol/L)	49.4 (20.1-125.3)	78.9 (15.1-423.7)	
PCR (mg/L)	1.2 (0.0-8.2)	27.6 (2.3-272.2)	
MCP-1 (pg/mL)	400.2 (243.6-795.5)	586.5 (285.1-1184.8)	
Actividad Paraoxonasa (U/L)	623.0 (175.3-656.5)	175.3 (83.5-380.1)	
Actividad Lactonasa (U/L)	5.9 (3.3-13.6)	3.1 (1.1-6.3)	
Concentracón PON1 (mg/L)	84.4 (45.1-277.8)	27.1 (13.7-63.4)	

Los resultados de las curvas ROC de los diferentes marcadores se muestran a continuación:

	AUC	95% CI
Isoprostanos (pg/mL)	0.997	0.995-1.00
HDL-C (mmol/L)	0.82	0.773-0.967
Insulina (pmol/L)	0.642	0.568-0.716
PCR (mg/L)	0.952	0.927-0.977
MCP-1 (pg/mL)	0.762	0.693-0.832
Actividad Paraoxonasa (U/L)	0.846	0.805-0.888
Actividad Lactonasa (U/L)	0.89	0.856-0.923
Concentracón PON1 (mg/L)	0.963	0.948-0.978

Todas las correlaciones entre las medidas de isoprostanos y los diferentes marcadores de EAP mostraron significación estadística ($P < 0.001$).

CONCLUSIONES:

La determinación de la concentración sérica de 8-isoprostanos es un marcador excelente de EAP, con una sensibilidad y especificidad diagnósticas muy elevadas y claramente superiores a las de los otros marcadores estudiados.

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EMERGING SERUM MARKERS IN PERIPHERAL ARTERY DISEASE

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Background:

The specificity and sensitivity of routine laboratory markers for the diagnosis of peripheral artery disease (PAD) are limited.

This study aimed to investigate the diagnostic capacity of the monocyte chemoattractant protein-1 (MCP-1), paraoxonase-1 (PON-1), carbonylated proteins and isoprostanes, (whose main characteristic is its duality to estimate the amount of oxidative stress and inflammation) in the diagnosis of PAD.

Methods:

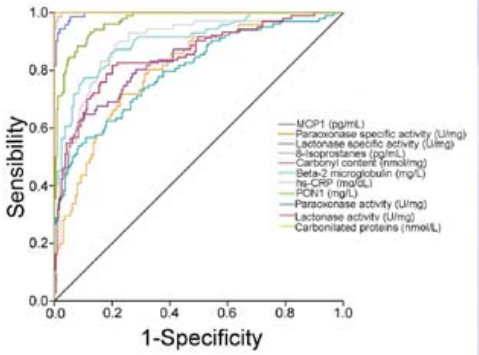
We investigated 115 male patients between 55 and 80 years, with clinically diagnosed PAD, and on which appropriate demographic and clinical characteristics were collected. We used as a control group samples from 300 healthy volunteers.

The circulating levels of C-reactive protein (hs-CRP) and beta-2 microglobulin were measured in a Modular P (Roche Diagnostics) automated analyzer. The concentration of MCP-1 was analyzed by ELISA. PON1 lactonase activity was done by measuring the ability of hydrolysis of 5-tiobutil butyrolactone, and PON1 paraoxonase activity, by measuring the hydrolysis of paraoxon. PON-1 concentration was determined by a ELISA based in mouse polyclonal antibodies against specific structures of PON1. Isoprostane concentration were determined by ELISA and and carbonylated proteins by photometry (Cayman® Chemical).

Results:

We observed significant increases in hs-CRP, beta-2-microglobulin, MCP-1, isoprostanes and carbonylated proteins, and significant decreases in all the PON1-related variables in PAD patients compared to controls (P<0.001).
The areas-under the curve of the receiver-operating characteristics curves (AUROC) were:

	AUROC
PON1 concentration	0,966
PON1 PARAOXONASE Activity	0,717
PON1 LACTONASE Activity	0,856
MCP-1	0,993
PARAOXONASE specific activity	0,782
LACTONONASE specific activity	0,780
ISOPROSTANES	0,999
CARBONILATED PROTEINS	0,756
CARBONYL CONTENT	0,756
B2-MICROGLOBULIN	0,899
hS-PCR	0,873



Conclusions:

isoprostanes showed a very high diagnostic accuracy in the diagnosis of PAD and may be considered as very promising new serum biomarkers of this disease.

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GALECTIN-3 APPLICATION IN PERIPHERAL ARTERY DISEASE

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Background:

The specificity and sensitivity of routine laboratory mar kers for the diagnosis of peripheral artery disease (PAD) are limited. Galectin-3 has an important role in cell cell adhesion, cell matrix interactions, macrophage activation and angiogenesis.

This study aimed to investigate the PAD diagnostic capacity of the galectin-3 compared to Chemokine (CC motif) ligand 2 (CCL2), paraoxonase1 (PON1), carbonyl content and isoprostanes, whose main characteristic is its duality to estimate the amount of oxidative stress and inflammation.

Methods:

We invest igated 86 patients, 18 female and 68 male, between 42 and 89 years, with clinically diagnosed PAD. Their appropriate demographic and clinical characteristics were collected. We used as a control group samples from 72 healthy volunteers, 25 female and 72 male, between 58 and 79 years.

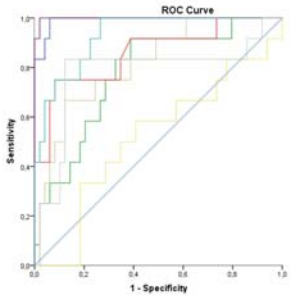
The circulating levels of C reactive protein measured by high sesitivity method (hs-CRP) and beta-2 microglobulin were measured in a Modular P (Roche Diagnostics)automated analyzer. The concentration of CCL2, PON1, isoprostanes (Cayman® Chemical) and galectin-3 (R&D® system) were analyzed by ELISA. PON1 lactonase activity was done by measuring the ability of hydrolysis of 5-tiobuitl butyrolactone, and PON1 paraoxonase activity, by measuring the hydrolysis of paraoxon. Carbonyl content was determined by a photometric assay (Cayman® Chemical).

Results:

We observed significant increases in hs-CRP, beta-2-microglobulin, CCL2, isoprostanes, carbonyl content and galectin-3 in PAD patients, and significant decrease in all PON1related variables in (P<0.001).

The areas-under the curve of the receiver-operating characteristics curves were:

	PAD
PON1 concentration	0,969
CCL2	0,992
PARAOXONASE specific activity	0,791
LACTONONASE specific activity	0,828
ISOPROSTANES	0,999
CARBONYL CONTENT	0,699
B2-MICROGLOBULIN	0,834
hS-PCR	0,805
Galectin-3	0,776



Conclusions:

Galectin-3 does not have as good diagnostic accuracy as Isoprostanes, but it may be considered as an interesting biomarker for PAD because of its near application in clinical laboratories.

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