Mecanismos de la enfermedad cardiovascular en la Enfermedad Pulmonar Obstructiva Crónica: papel de la quimiocina CCL18 y proteínas de matriz extracelular

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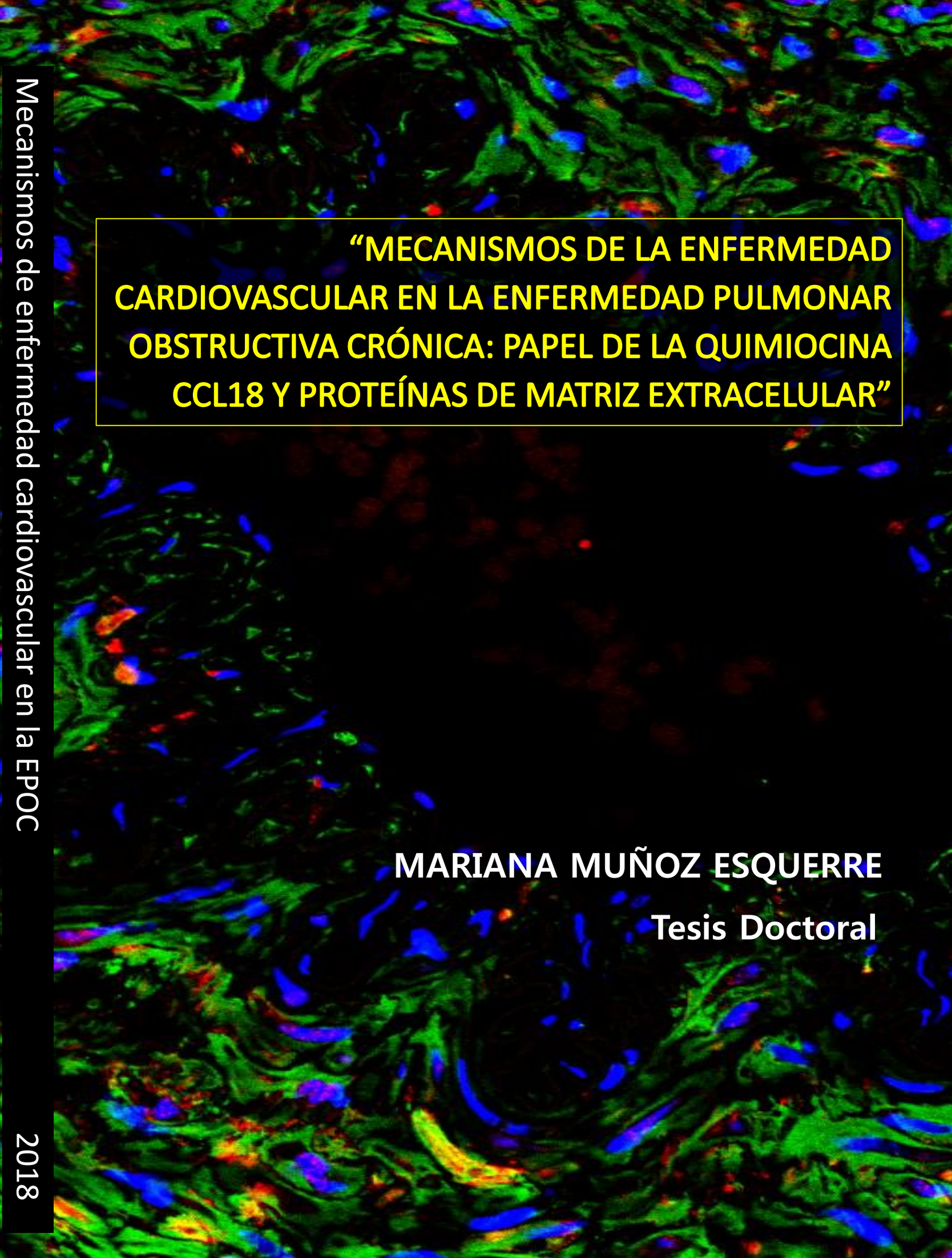

“MECANISMOS DE LA ENFERMEDAD CARDIOVASCULAR EN LA ENFERMEDAD PULMONAR OBSTRUCTIVA CRÓNICA: PAPEL DE LA QUIMIOCINA CCL18 Y PROTEÍNAS DE MATRIZ EXTRACELULAR”

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Mecanismos de enfermedad cardiovascular en la EPOC

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"MECANISMOS DE LA ENFERMEDAD CARDIOVASCULAR EN LA ENFERMEDAD PULMONAR OBSTRUCTIVA CRÓNICA: PAPEL DE LA QUIMIOCINA CCL18 Y PROTEÍNAS DE MATRIZ EXTRACELULAR"

Tesis doctoral presentada por

MARIANA MERCEDES MUÑOZ ESQUERRE

para optar al grado de Doctora en Medicina

Directores: Dra. SALUD SANTOS PÉREZ y Dr. JORDI DORCA SARGATAL

Barcelona, 11 de Junio de 2018

La Dra. Salud Santos Pérez, Facultativa Adjunta del Servicio de Neumología del Hospital Universitario de Bellvitge y Profesora de la Facultad de Medicina de la Universidad de Barcelona, y el Dr. Jordi Dorca Sargatal, Jefe del Servicio de Neumología del Hospital Universitario de Bellvitge, Jefe del Grupo de Investigación de Neumología del IDIBELL y Profesor de la Facultad de Medicina de la Universidad de Barcelona.

HACEN CONSTAR

Que Mariana Mercedes Muñoz Esquerre, licenciada en Medicina, ha realizado bajo su dirección el trabajo de investigación para elaborar su Tesis Doctoral titulada **"Mecanismos de la enfermedad cardiovascular en la Enfermedad Pulmonar Obstructiva Crónica: papel de la quimiocina CCL18 y proteínas de matriz extracelular"**, la consideran finalizada y autorizan su presentación para ser defendida ante el tribunal que corresponda para optar al Grado de Doctora en Medicina

En Barcelona, 1 de Junio de 2018

Dra. Salud Santos Pérez

Dr. Jordi Dorca Sargatal

A mis niños: el grande y los dos pequeños, por la fortuna de tenerlos,

mi mundo es un lugar infinitamente mejor con ustedes

A mis padres y hermanos, por ser la base del amor y la unión familiar

Ama sua, ama llulla, ama quella

(no seas ladrón, no seas mentiroso, no seas flojo)

Principio inca

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CONTENIDO

LISTADO DE ABREVIATURAS

AAS	Ácido acetilsalicílico
AEPOC	Agudizaciones de la Enfermedad pulmonar obstructiva crónica
CMLs	Células musculares lisas
EPOC	Enfermedad pulmonar obstructiva crónica
FN	Fibronectina
HTP	Hipertensión pulmonar
IL	Interleucina
MEC	Matriz extracelular
PARC	Pulmonary and activation-regulated chemokine
PCR	Proteína C Reactiva
PRI	Índice de reactividad $P2Y_{12}$
VASP	Fosfoproteína estimulada por vasodilatador
VEMS	Volumen espiratorio máximo en el primer segundo
TAC	Tomografía axial computarizada
TNC	Tenascina-C

1. INTRODUCCIÓN

La enfermedad pulmonar obstructiva crónica (EPOC) es la enfermedad respiratoria crónica con mayor prevalencia e impacto, tanto en la salud de la población como en el sistema sanitario, de tal manera que es considerada en la actualidad como un problema principal de salud mundial, representando la cuarta causa de muerte a nivel global.[1–3] Se prevé que en los próximos años, la EPOC se convierta en la tercera causa de muerte, detrás de las enfermedades coronarias y cerebrovasculares[4] y la quinta causa de discapacidad, afectando aproximadamente al 10% de la población mayor de 45 años.[5] Se estima además, un incremento de la prevalencia en las próximas décadas debido a la exposición continua a los factores de riesgo, principalmente al tabaco y al envejecimiento de la población.[4]

La EPOC es una enfermedad compleja dada su presentación heterogénea y difícil de definir o clasificar tan sólo por un parámetro funcional pulmonar, como es el volumen espiratorio máximo en el primer segundo (VEMS). Los cambios patológicos a nivel pulmonar afectan principalmente a las vías aéreas, al parénquima pulmonar y a las estructuras vasculares.[6] Como consecuencia clínica de estas alteraciones, se observan distintos grados de limitación al flujo aéreo, de enfisema pulmonar y cambios vasculares pulmonares.[7–9] Un mecanismo patogénico común en el desarrollo de estas alteraciones es la presencia de inflamación crónica.[10,11] Esta inflamación observada en los pacientes con EPOC, va más allá del proceso inflamatorio normal como respuesta a la inhalación de irritantes respiratorios, como el humo

del tabaco; esta inflamación es aberrante, amplificada y persistente, incluso tiempo después del cese tabáquico.[10,12]

Este proceso inflamatorio no sólo se observa a nivel local pulmonar, sino también a nivel sistémico,[13] por lo que se cree que estaría relacionado con algunas de las comorbilidades y/o con otros efectos extrapulmonares que se describen en pacientes con EPOC: enfermedades cardiovasculares, síndrome metabólico, cáncer de pulmón, disfunción muscular esquelética y desnutrición, entre otras.[10,14–16] Dentro de las comorbilidades asociadas a la EPOC, la enfermedad cardiovascular es la más prevalente y con mayor impacto en la mortalidad.[17] De hecho, la mortalidad de los pacientes con EPOC por causa cardiovascular llega a ser del 25 % en pacientes jóvenes y menos graves desde el punto de vista funcional, es decir, con limitación obstructiva leve a moderada al flujo aéreo.[16]

1.1 La EPOC como factor de riesgo cardiovascular

Se reconocen diversos factores de riesgo cardiovascular, llamados factores de riesgo clásicos, entre ellos la hipercolesterolemia, la hipertensión arterial, la diabetes y el tabaquismo.[18] La diabetes y la hipertensión arterial, son patologías muy prevalentes en la población general, sobre todo en los mayores de 60 años. De hecho, cuando se analiza la prevalencia de estas dos patologías en pacientes con EPOC, se observa una mayor prevalencia respecto a la población general y a medida que la gravedad aumenta (medida por categorías GOLD 0-4) el riesgo de padecer estas comorbilidades es aún mayor.[19] El

tabaquismo, a su vez es el principal factor de riesgo en nuestro medio para desarrollar una EPOC;[3] por tanto es muy difícil desligar el efecto directo del consumo de tabaco sobre el desarrollo de la patología cardiovascular en estos pacientes. Sin embargo, cuando se analizan todas estas variables en estudios longitudinales poblacionales, el riesgo cardiovascular asociado a la presencia de EPOC, es independiente de factores como la edad avanzada, la presencia de hipertensión arterial, diabetes, dislipidemia o el grado de tabaquismo.[20]

Por otro lado, se ha descrito que los pacientes con enfermedad cardiovascular y EPOC presentan un peor pronóstico, teniendo al menos el doble de riesgo de mortalidad a corto y largo plazo que los sujetos sin EPOC.[21–23] Pero no sólo existe esta asociación de enfermedades con impacto pronóstico desfavorable, sino que además la gravedad de la enfermedad respiratoria impacta en el pronóstico cardiovascular, siendo el VEMS y el índice VEMS/capacidad vital forzada, predictores independientes de riesgo cardiovascular y mortalidad.[23–25] Entre las entidades cardiovasculares descritas se encuentran fundamentalmente la cardiopatía isquémica, la insuficiencia cardiaca, arritmias cardiacas, ictus, enfermedad vascular periférica y la hipertensión arterial.[20,22]

Cardiopatía isquémica en pacientes con EPOC

La cardiopatía isquémica es una de las enfermedades cardiovasculares más relevantes, debido a su alta prevalencia y su gran impacto sobre la salud.[26]

Investigaciones de base poblacional determinan que padecer una EPOC aumenta el riesgo de enfermedad coronaria independientemente de otros factores de riesgo cardiovascular.[27] Adicionalmente, los pacientes con EPOC presentan una prevalencia de enfermedad coronaria del 33.6% versus el 27.1% en sujetos sin EPOC.[28] A mayor severidad de la enfermedad respiratoria, existe una mayor incidencia de eventos coronarios fatales,[16,29] presentando un riesgo de muerte cinco veces mayor los pacientes en el quintil inferior del VEMS respecto a los quintiles superiores.[23]

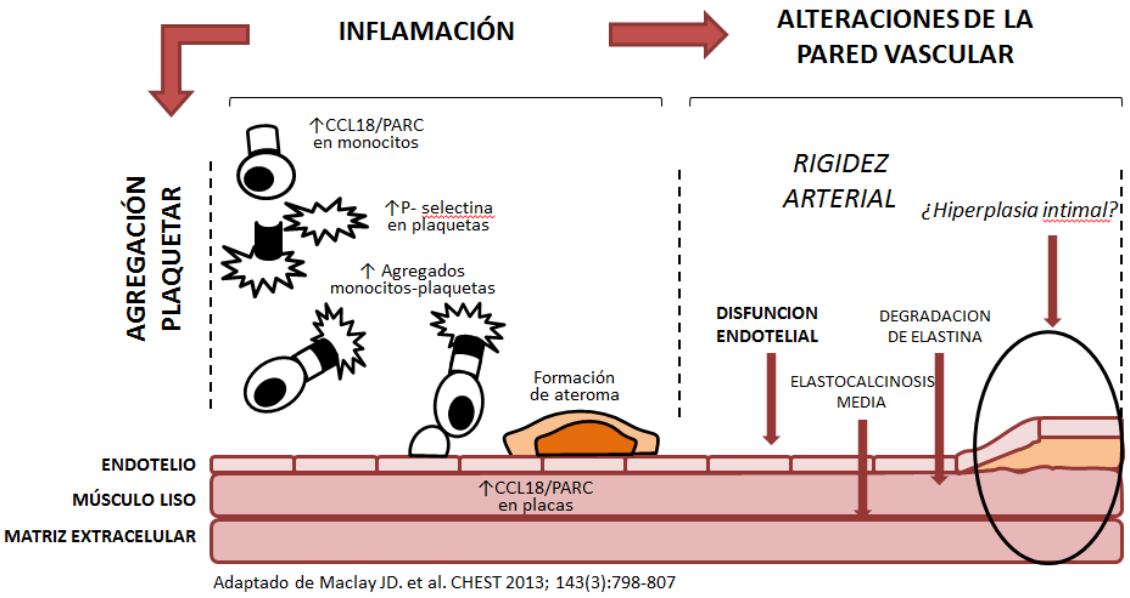
Es importante destacar que los pacientes con EPOC no sólo presentan un peor pronóstico cardiovascular a corto plazo, sino una mortalidad de dos a cuatro veces mayor en el seguimiento a largo plazo después de sufrir un infarto agudo de miocardio comparado con sujetos sin EPOC.[21,22,30,31] En resumen, padecer una EPOC incrementa el riesgo de sufrir una enfermedad coronaria isquémica con un peor pronóstico a corto y largo plazo.

1.2 Mecanismos de aterotrombosis asociados a la EPOC

Varios mecanismos han sido propuestos como posibles responsables del desarrollo de aterotrombosis causantes de la patología cardiovascular en los pacientes con EPOC. Entre ellos tenemos la inflamación sistémica, el estrés oxidativo, el estado de hipercoagulabilidad y la agregación plaquetar. Otros factores como las anormalidades de la pared vascular, el envejecimiento acelerado y el desequilibrio proteasa/antiproteasa formar parte también de las

hipótesis sobre dicha patogenia.[32] Lo que sí está descrito, es que todos estos mecanismos son complejos, no muy bien conocidos y posiblemente inter-relacionados entre sí. Sin embargo, existe una mayor evidencia en que el proceso inflamatorio sistémico podría ser el desencadenante o agravante en la formación de placas de ateroma, en las anormalidades de la pared vascular, así como en el aumento de la hipercoagulabilidad y de la activación plaquetar, lo que finalmente explicaría el desarrollo de eventos cardiovasculares en los pacientes con EPOC (Figura 1).[32]

Figura 1. Mecanismos posibles de aterotrombosis asociados a la EPOC



Inflamación sistémica

La aterosclerosis es la principal causa subyacente de enfermedad arterial coronaria.[33] En lo que respecta a su patogénesis, se conoce que es un proceso complejo, con algunos mecanismos específicos por los cuales no sólo la hipercolesterolemia, sino también la presencia de inflamación puede alterar la biología de la pared vascular y participar en el desarrollo de la enfermedad cardiovascular, tanto en el inicio, como en la formación y ruptura de la placa de ateroma.[33,34] De este modo, existen elementos de la inmunidad innata y de la inmunidad adaptativa del sujeto que van a promover un proceso inflamatorio asociado a la arterioesclerosis.[35]

Dentro de la inmunidad innata, son las células fagocitarias mononucleares las que tienen el rol principal, siendo inicialmente reclutadas de la circulación para luego unirse a la capa de células endoteliales. Una vez en la pared arterial diversos mediadores o citocinas, llamadas específicamente quimiocinas, son las encargadas de dirigir la migración de estos monocitos a la capa íntima para formar parte de las células espumosas dando inicio al desarrollo de la arteriosclerosis.[33,35] Los monocitos residentes en las lesiones arterioscleróticas a su vez, son capaces de propagar la respuesta innata, expresando citocinas proinflamatorias y otros mediadores como las metaloproteinasas de matriz.[33]

Existe también, una aportación de la inmunidad adaptativa al proceso de arteriosclerosis. Por ejemplo, las células dendríticas intervendrían como células presentadoras de antígenos ante los linfocitos T, los cuales promoverían a su

vez una respuesta celular inmune en la placa de ateroma.[36] Además, todas las subclases funcionales de células T podrían intervenir en mayor o menor grado en los procesos de arteriosclerosis, amplificando vías proinflamatorias (función Th1, posiblemente Th17 y natural killer) y aumentando el crecimiento y transformación de las placas de ateroma (células T CD8 y células T reguladoras).[33,36,37] Existe sin embargo, controversias sobre la función Th2 (que clásicamente modula la inflamación y promoción de la inmunidad humoral), la cual podría participar en la formación de los aneurismas de la pared arterial y en la formación de placas de ateroma.[38,39] Por el contrario, actuaría atenuando el desarrollo de la arteriosclerosis, debido a la producción de anticuerpos por ejemplo contra las lipoproteínas de baja densidad.[40,41]

Anormalidades de la pared vascular sistémica y pulmonar en la EPOC

A nivel pulmonar se ha descrito que la inflamación sostenida y desencadenada por la exposición al humo del tabaco podría promover alteraciones en la composición y estructura de la pared vascular.[11] Estas alteraciones conocidas como remodelado vascular, se han estudiado exhaustivamente en pacientes con EPOC y en hipertensión pulmonar de otro origen, describiendo un proceso que se inicia con la proliferación y migración de las células musculares lisas de la capa media hacia la capa intima, induciendo una hiperplasia intimal con la consecuente disminución del calibre de la luz vascular,[42,43] con depósito de proteínas de la matriz extracelular (MEC) y la

existencia de un infiltrado inflamatorio caracterizado por linfocitos T CD8+ en la capa adventicia.[44–46]

A nivel sistémico, las principales alteraciones vasculares encontradas en pacientes con EPOC se relacionan con la función (disfunción endotelial y rigidez arterial) y la estructura de la pared vascular.[47,48]

Disfunción Endotelial y rigidez arterial

Los análisis de la función endotelial provienen de observaciones indirectas, mediante ultrasonidos, y lo descrito es una disminución en la liberación endotelial del óxido nítrico, que es un potente vasodilatador, como parte de la respuesta vasomotora a la oclusión arterial provocada. Esta disfunción endotelial se asocia a una mayor obstrucción bronquial y la severidad del enfisema pulmonar, así como a niveles altos de inflamación sistémica en pacientes con EPOC.[47,49,50.]

Así mismo, los estudios sobre la rigidez arterial de las arterias sistémicas de gran calibre, como son la aorta y las arterias carótidas, analizando la velocidad de la onda del pulso (*pulse wave velocity*), muestran un incremento en la rigidez arterial en los pacientes con EPOC, comparado con sujetos fumadores. Es de destacar que esta medida tiene un valor predictivo de eventos cardiovasculares en la población general y en pacientes con cardiopatía isquémica. Además, se describe una asociación entre la rigidez arterial y la

severidad de la limitación al flujo aéreo, el grado de enfisema evaluado por tomografía axial computarizada (TAC) y el grado de inflamación sistémica medida por los niveles de interleucina-6 (IL-6) y proteína C-reactiva (PCR) circulantes.[51–54]

Arteriosclerosis subclínica

Estudios morfométricos realizados mediante ultrasonografía ponen de manifiesto un mayor engrosamiento valorado por el índice íntima-media a nivel de las arterias carotídeas, en sujetos con EPOC respecto a controles.[55–57] De este mismo modo, mediante estudios por TAC, también se ha descrito una mayor presencia de placas calcificadas a nivel coronario, carotideo y aórtico en pacientes con EPOC respecto a fumadores y asociadas a niveles altos de inflamación medidos por la PCR.[58,59]

Cabe destacar que no existen estudios que analicen directamente los cambios vasculares como el remodelado de las arterias sistémicas de los pacientes con EPOC, ni su relación con el remodelado vascular pulmonar y las variables clínicas asociadas a este remodelado. Por otro lado, en este contexto de interacciones entre la función, estructura vascular y la inflamación (sistémica y local) tendríamos que considerar a las quimiocinas como moléculas reguladoras de estos procesos.

Expresión de las quimiocinas en patología vascular

Las quimiocinas o citocinas quimiotácticas son una gran familia de proteínas que actúan como mediadores importantes de la hematopoyesis y migración leucocitaria durante los procesos inflamatorios. Forman parte del nexo de unión entre la inmunidad innata y adquirida, y juegan un papel importante en la diferenciación y activación celular.[60] Las células estructurales también participan de manera activa en la producción de quimiocinas y en la expresión de sus receptores, a través de los cuales controlan los eventos inflamatorios locales.[61] La participación de las quimiocinas en el desarrollo de la arteriosclerosis ha sido descrita en diversos estudios y no cabe duda de la importancia de estos mediadores en el proceso de regulación inflamatoria que acompaña a la progresión de la arteriosclerosis y la desestabilización de la placa de ateroma.[62,63] Entre la gran familia de quimiocinas, en arterioesclerosis se ha observado la participación de dos tipos: el grupo CXC-quimiocinas (como la interleucina-8 o el péptido activador de neutrófilo) y el grupo de las CC-quimiocinas, como por ejemplo la proteína-1 quimioatrayente del monocito, CCL19 o CCL21, los cuales se expresan dentro de las lesiones ateroscleróticas junto con sus correspondientes receptores.[62]

Sin embargo, dentro de éste último grupo, podría ser de especial interés la quimiocina CCL18 (*CC-chemokine ligand 18*), que fue inicialmente denominada PARC (*pulmonary and activation-regulated chemokine*) debido a que se expresa constitutivamente en algunas líneas celulares humanas y predominantemente en el tejido pulmonar.[64,65] Es una quimiocina circulante que

juega un papel relevante en la reparación del daño tisular, localización de células mononucleares y en la respuesta inflamatoria.[66] Específicamente, CCL18 atrae linfocitos T, B y células dendríticas inmaduras derivadas de monocitos, siendo la mayor fuente celular de esta proteína, las células de linaje monocítico, incluidas las células dendríticas.[65]

En la patología cardiovascular, se ha demostrado que la expresión génica de CCL18 está aumentada en placas ateroescleróticas humanas provenientes de endarterectomías carotideas, localizándola por inmunohistoquímica en los macrófagos, monocitos y linfocitos.[67,68] Esta quimiocina se expresa particularmente en sitios de inestabilidad de la placa de ateroma.[68] Se postula que los monocitos previamente atraídos a la placa de ateroma por otras citocinas producirían CCL18, la cual atraería a su vez a linfocitos T para dar continuidad a la cascada inflamatoria en la pared arterial. Este acumulo de monocitos se observa durante el proceso de la arteriosclerosis y en cantidades proporcionales a la progresión de la enfermedad.[66] En este sentido, al ser un producto circulante y secretado por los monocitos activados, algunos autores la postulan como posible biomarcador sérico predictivo de nuevos eventos CV en pacientes con cardiopatía isquémica estable.[66]

Cabe destacar que en la EPOC, se han realizado diversos estudios sobre biomarcadores en grandes cohortes de pacientes, encontrando niveles séricos elevados de CCL18 en dichos pacientes comparado con controles fumadores y asociando este aumento de CCL18 a una mayor mortalidad por causa

cardiovascular.[69,70] **Aunque en la actualidad se desconoce la función biológica exacta que desarrolla CCL18 en muchos procesos, estos hallazgos incrementan el interés por conocer el papel de esta molécula en la fisiopatología de la EPOC y su asociación con la patología cardiovascular.**

Proteínas de la matriz extracelular en patología vascular

La síntesis de las proteínas de la MEC de la pared vascular y su metabolismo son procesos estrictamente regulados con el objetivo de mantener en homeostasis la función y estructura de los vasos sanguíneos. Cualquier daño mecánico, químico o enzimático puede alterar la estructura y promover cambios en la composición de esta matriz, lo cual puede traer consigo procesos de remodelado y alteraciones vasculares asociadas.[71]

Estas proteínas de la MEC son una compleja red de proteínas secretadas por las células que contribuyen al desarrollo y comportamiento de los tejidos, regulando por ejemplo el endotelio y el comportamiento de las células musculares lisas, controlando su crecimiento, diferenciación y supervivencia.[72] En concreto, la Fibronectina (FN) y la Tenascina C (TNC) podrían tener un papel relevante en el remodelado vascular pulmonar asociado a la EPOC como se explica a continuación.

La FN, es una glicoproteína hallada en la matriz extracelular de los tejidos (forma celular) y en el plasma. En el tejido es producida por diversos tipos de

células incluidas las células musculares lisas, fibroblastos y miofibroblastos y ampliamente distribuidas en la MEC.[71] Su función a nivel de la vasculatura es el de modificar, detectar y reaccionar ante cambios mecánicos, elásticos y de estrés a nivel de la pared arterial juntos con las CMLs.[43] Además se ha descrito que puede modular la infiltración leucocitaria, la expresión de moléculas de adhesión, la proliferación celular y el fenotipo de CMLs, es decir, todos los mecanismos involucrados en el remodelado de la pared arterial.[73] Existen dos variantes génicas ED-A y ED-B, las dos están altamente expresadas en tejido vascular embriogénico y ciertos procesos patogénicos, facilitando la diferenciación fenotípica de CMLs. ED-A FN estaría mayormente asociada a procesos fibrogénicos secundarios al daño pulmonar.[74,75]

La TNC, es otra glicoproteína de matriz expresada durante la morfogénesis y durante la reparación de tejidos, por tanto no expresadas constitutivamente en el tejido sano de adultos.[76,77] In vitro, TNC es capaz de promover la angiogénesis y liberar citocinas pro-inflamatorias y metaloproteinasas de matriz.[78] En estudios animales y en tejido humano, la TNC tendría un rol importante en el desarrollo de la hiperplasia intimal secundaria al daño vascular (angioplastia, arteriotomia, *stents* o injertos arteriales),[79,80] en el contexto de la hipertensión pulmonar primaria[72,81] y en el aumento de la fibrosis miocárdica post-infarto de miocardio.[82]

En la patología vascular causante de la hipertensión pulmonar (HTP) primaria, la cual se caracteriza por un remodelado de la pared vascular, se ha

visto que estas dos proteínas se expresan con mayor intensidad a medida que las lesiones vasculares progresan y que la proliferación de CMLs sería dependiente de TNC y que la migración podría depender de FN.[46]

En lo que respecta a la EPOC, concretamente los resultados se pueden resumir, como un aumento de la expresión proteica de FN en el endotelio vascular y en la mucosa bronquial.[83] La expresión génica pulmonar de FN en etapas tempranas de la EPOC está aumentada y a medida que progresa el daño pulmonar, disminuye su expresión pre-transcricpcional.[84] En el caso de la TNC, existe una asociación entre el aumento de la expresión a nivel bronquial y el grado de remodelado de la pared bronquial en pacientes con EPOC.[83,85,86] Sin embargo, en estudios que exploran la regulación génica en el contexto de HTP asociada a la EPOC, la TNC concretamente no se sobreexpresa, como sí lo hace en la HTP asociada a la fibrosis pulmonar.[87] **Por lo tanto, no existe hasta la fecha una evidencia concordante sobre la expresión de dichas glicoproteínas en el contexto del remodelado vascular pulmonar y en la HTP asociada a la EPOC.**

Activación y agregación plaquetar

Los pacientes con EPOC en el transcurso de su enfermedad pueden presentar diversos eventos respiratorios con empeoramiento de los síntomas y necesidad de cambios en la terapia habitual. Estos eventos son conocidos como agudizaciones o exacerbaciones de la EPOC (AEPOC) y principalmente son desencadenadas por infecciones agudas del tracto respiratorio.[1] Estas agudizaciones juegan un papel pronóstico importante: producen pérdida

acelerada de la función pulmonar, además de un aumento considerable en la tasa de mortalidad.[16] Un aspecto relevante, es la observación del aumento de los eventos cardiovasculares durante esta fase, habiéndose reportado un incremento de 2 a 3 veces el riesgo de infarto de miocardio durante los cinco primeros días del episodio de agudización.[88]

La inflamación sistémica, además de ser un determinante conocido en el desarrollo de la arterioesclerosis, lo es también de los eventos aterotrombóticos, debido a que contribuye a la activación plaquetar.[34,63] Las plaquetas, son un elemento clave en el desarrollo de estas complicaciones aterotrombóticas derivadas de la aterosclerosis, y su acción se podría resumir en tres fases: adhesión, activación y finalmente, agregación.[89,90] Tras la rotura de la placa de ateroma, son liberadas ciertas substancias que promoverán el reclutamiento y la adhesión de las plaquetas circulantes a esta zona de daño endotelial, lo que es seguido de la activación y agregación de las mismas.[91]

En los pacientes con EPOC, la inflamación persistente se agrava durante estas agudizaciones de la enfermedad,[92] lo cual se postula que podría desencadenar la activación y agregación plaquetar, proceso fundamental en el desarrollo de un evento isquémico agudo.[90] En este sentido, se ha descrito un aumento significativo de los agregados monocito-plaquetarios y de la expresión plaquetaria de P-selectina (un marcador de activación plaquetar) durante las exacerbaciones de la EPOC.[93] Sin embargo, estos dos parámetros son poco específicos y no valoran estrictamente la función plaquetar ni sus vías de

activación.[93] **Por tanto, hasta la fecha, no existe ningún estudio que haya demostrado mediante pruebas de función plaquetar con capacidad predictiva demostrada de eventos clínicos (en pacientes con cardiopatía isquémica) el impacto que tienen las agudizaciones sobre el aumento de esta reactividad plaquetar. Es importante señalar, que tampoco existen datos sobre el efecto de la terapia antiagregante plaquetaria durante las AEPOC.** De confirmarse dicho aumento en la reactividad plaquetar, esto contribuiría a explicar el elevado riesgo de eventos isquémicos en pacientes con EPOC agudizada y plantearía nuevos enfoques terapéuticos en este grupo de pacientes.

2. HIPÓTESIS

La hipótesis principal de esta tesis doctoral es que los pacientes con EPOC presentan un mayor riesgo cardiovascular que los pacientes fumadores sin EPOC debido a alteraciones vasculares como el remodelado de las arterias, tanto pulmonares como sistémicas, donde la quimiocina CCL18/PARC y las proteínas de la matriz extracelular Fibronectina y Tenascina-C juegan un papel patogénico. Dicho riesgo cardiovascular aumenta durante la fase de agudización debido en parte a una mayor agregabilidad plaquetar asociada al aumento del proceso inflamatorio sistémico.

3. OBJETIVOS

El objetivo general de esta tesis es profundizar en el conocimiento de algunos mecanismos de aterotrombosis asociados a la EPOC, analizando la estructura de la pared vascular y los posibles mediadores implicados, tanto en la fase estable como durante las agudizaciones. Para la consecución de este objetivo se han realizado los estudios incluidos en esta tesis, con los siguientes objetivos específicos:

1. Evaluar el remodelado de las arterias sistémicas y pulmonares de pacientes con EPOC, comparado con fumadores sin EPOC y controles, así como describir la correlación existente entre los cambios simultáneos a nivel sistémico y pulmonar y los factores clínicos asociados al remodelado de la pared vascular.

2. Analizar la expresión vascular sistémica y pulmonar de la quimiocina PARC/CCL18 y su relación con el remodelado vascular de pacientes con y sin EPOC.

3. Analizar la expresión génica, caracterización celular y contenido proteico pulmonar de FN y TNC en relación al remodelado vascular pulmonar en pacientes con y sin EPOC.

4. Determinar el impacto que tienen las agudizaciones de la EPOC sobre la reactividad plaquetar, con independencia del tratamiento antiagregante recibido, y valorar la asociación entre dicha agregabilidad plaquetar y el grado de inflamación que desarrollan durante la fase aguda de la enfermedad.

4. PUBLICACIONES

Estudio I. Systemic and Pulmonary Vascular Remodelling in Chronic Obstructive Pulmonary Disease

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RESEARCH ARTICLE

Systemic and Pulmonary Vascular Remodelling in Chronic Obstructive Pulmonary Disease

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Abstract

Background

Chronic Obstructive Pulmonary Disease (COPD) is associated with subclinical systemic atherosclerosis and pulmonary vascular remodelling characterized by intimal hyperplasia and luminal narrowing. We aimed to determine differences in the intimal thickening of systemic and pulmonary arteries in COPD subjects and smokers. Secondary aims include comparisons with a non-smokers group; determining the clinical variables associated with systemic and pulmonary intimal thickening, and the correlations between systemic and pulmonary remodelling changes.

Methods

All consecutive subjects undergoing lung resection were included and divided into 3 groups: 1) COPD, 2) smokers, and 3) non-smokers. Sections of the 5th intercostal artery and muscular pulmonary arteries were measured by histo-morphometry. Four parameters of intimal thickening were evaluated: 1) percentage of intimal area (%IA), 2) percentage of luminal narrowing, 3) intimal thickness index, and 4) intima-to-media ratio.

Results

In the adjusted analysis, the systemic arteries of COPD subjects showed greater intimal thickening (%IA) than those of smokers (15.6±1.5% vs. 14.2±1.6%, p = 0.038). In the pulmonary arteries, significant differences were observed for %IA between the 2 groups (37.3 ±2.2% vs. 29.3±2.3%, p = 0.016). Among clinical factors, metabolic syndrome, gender and COPD status were associated with the systemic intimal thickening, while only COPD status was associated with pulmonary intimal thickening. A correlation between the %IA of the systemic and pulmonary arteries was observed (Spearman's rho = 0.46, p = 0.008).

sponsors did not have any role in the study design, collection, analysis and interpretation of data, or in writing the report.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

Greater intimal thickening in systemic and pulmonary arteries is observed in COPD patients than in smokers. There is a correlation between systemic and pulmonary vascular remodelling in the overall population.

Introduction

The most important comorbidity associated with chronic obstructive pulmonary disease (COPD), due to its impact on prognosis and mortality, is cardiovascular disease (CVD).[1–2] In this setting, previous studies suggest that COPD subjects have an increased risk of ischemic heart disease independent of smoking, age or gender.[3–5] However, the underlying mechanisms of this frequent association (between COPD and CVD) have not been completely elucidated.[4] Although the pathogenesis of atherosclerosis is complex, low-grade systemic inflammation, which is present in COPD and in CVD, could be one of the centrepiece events leading to systemic vascular remodelling and plaque formation.[3] Moreover, the remodelling of pulmonary vessels is a well-recognized finding in COPD.[6–7] This process is characterized by the migration and proliferation of vascular smooth muscle cells, inducing intimal hyperplasia and, therefore, subsequent luminal narrowing.[7] These pulmonary changes are mainly caused by sustained inflammatory process triggered by smoke exposure.[8]

However, to date, to the best of our knowledge, no previous histological study has evaluated vascular remodelling changes, such as the intimal thickening of the systemic arteries of COPD subjects, and its relationship with the presence of pulmonary intimal hyperplasia. The principal aim of study was to determine differences in intimal thickening in terms of the percentage of intimal area (%IA) of systemic arteries in COPD subjects and smokers. Secondary aims include an evaluation of differences in other intimal thickening parameters, all parameter comparisons with a non-smoker group, the determination of the variables associated with systemic and pulmonary intimal thickening and an evaluation of the correlation between systemic and pulmonary changes in the overall population.

Methods

Population

This was a prospective investigation, conducted in consecutive subjects who required lung resection for the treatment of lung cancer recruited from the Department of Pulmonary Medicine of University Hospital of Bellvitge (L'Hospitalet de Llobregat, Spain). Demographic and clinical data were obtained from patients' medical records. A preoperative pulmonary function test was performed in all subjects. Patients were divided into three groups according to their smoking history and pulmonary function tests: 1) COPD subjects (all of whom were current or former smokers with airflow limitation), 2) smokers (current or former smokers with normal lung function), and 3) non-smokers (never-smokers). Exclusion criteria were the presence of any pulmonary disease other than COPD and previous treatment with chemotherapy or radiotherapy regimens or previous lung surgery. The definition of COPD was established following the current guidelines.[1] The study was approved by the local ethics committee "Comitè Ètic d' Investigació Clínica del Hospital de Bellvitge, N° PR006/11", and performed in accordance with the Declaration of Helsinki. All patients signed an informed consent form.

Sample collection

To evaluate systemic circulation, sections of the 5th posterior intercostal (IC) artery (1–1.5cm in length) were taken during the thoracotomy incision. To assess pulmonary circulation, lung samples were obtained from the piece of lung resection, as far away as possible from the tumour, and muscular pulmonary arteries with an external diameter between 100 to 500 μm were considered in the analyses. Both tissues (IC artery and lung samples) were fixed overnight in 4% paraformaldehyde following established methods of fixation and preparation of samples for morphometry.[9] Venous blood samples were collected from all subjects. Fasting blood glucose (FBG), total cholesterol, triglycerides, high-density lipoprotein cholesterol (cHDL), low-density lipoprotein cholesterol (cLDL), and blood cell counts were determined with standard laboratory methods. Metabolic syndrome (MetS) was defined as the presence of three or more of the metabolic parameters in accordance with current criteria as detailed in S1 File.[10]

Morphometric analysis

Tissue sections were stained with elastin-orcein stain to localize elastin fibres and to differentiate the external elastic lamina (EEL) and internal elastic lamina (IEL) from all arteries (Fig 1A). Only vessels with complete elastic laminas were evaluated. Using a digital micro-imaging device (Leica DMD108, Leica Microsystems GmbH- Germany), EEL and IEL and the inner aspect of the intimae were outlined as previously described [9] by two different observers working blindly with regard to the study groups. In addition, artery diameters and areas of lumen, IEL, intimal and medial layer were calculated (Fig 1B). In order to evaluate the effect of vascular contraction and tissue shrinkage during manipulation or fixation, an index of narrowing was estimated for each artery (S1 File).[11] In line with previous studies of vascular remodelling and atherosclerosis and following stereological methods for vascular evaluation,[9, 12–13] four methods were used to analyze the degree of intimal thickening: 1) percentage of intimal area

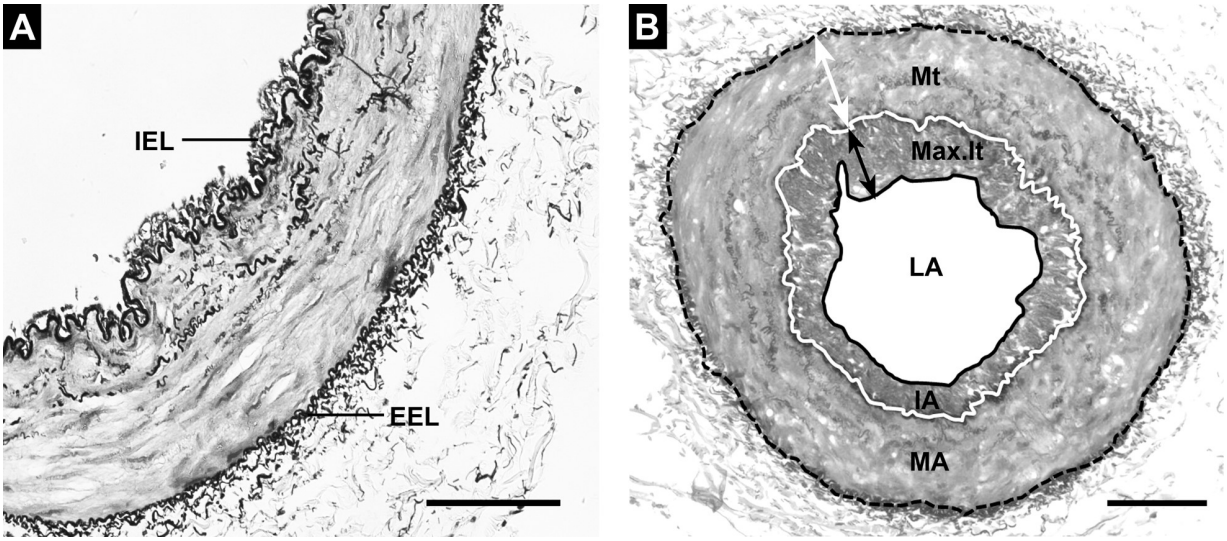

Fig 1. Histologic and morphometric analyses. A. Fifth intercostal artery showing almost no intimal thickness and muscular media layer. Note also the absence of elastic laminas except for the internal elastic lamina (IEL) and external elastic lamina (EEL). **B.** Methods used for morphometric analyses as described in the text. A solid white line represents the IEL and the discontinuous black line shows the EEL. The area enclosed by the solid black line is the lumen area (LA), the area enclosed by the solid white line is the combined lumen + intima area (IA) and the area enclosed by the discontinuous black line is the lumen + intima + media area (MA). A double-headed black arrow represents maximal intimal thickness (Max.It). The double-headed white arrow shows the medial thickening (Mt) at maximal intimal thickness. Elastin-orcein stain, scale bar = 100μm.

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(%IA = 100Xintimal area/measured total area or area encompassed by the EEL), 2) percentage of luminal narrowing (%LN = 100Xintimal area/IEL area) which consider IEL as the surface reference, 3) intimal thickness index (ITI = intimal area/medial area), and 4) intima-to-media ratio (IMR = width of intima at maximal intimal thickness/width of media at maximal intimal thickness).

Study endpoints and sample size calculation

The primary endpoint of this study was the difference in the %IA of systemic arteries in COPD patients compared to smokers. Assuming a standard deviation of 5(%), a sample size of 12 subjects per group was needed to detect a minimal difference between groups of 6%; with 80% power and a two-tailed p-value less than 0.05. Considering an approximate 20% dropout rate (e.g. inadequate samples for morphometric measurements), the inclusion of 15 subjects by group was allowed to ensure that data from 12 patients was available for analysis. Secondary endpoints included: 1) between-group comparisons for %LN, ITI and IMR of systemic arteries, 2) all parameter comparisons with a non-smoker group, 3) associations between clinical variables and %IA of systemic and pulmonary arteries, and 4) correlations between systemic and pulmonary remodelling parameters in the overall population.

Statistical methods

For baseline characteristics, continuous variables were expressed as mean±SD or by median and interquartile range, whether a normal distribution was assumed or not (Kolmogorov-Smirnov test), respectively. Comparisons of continuous variables were performed with the Student's t-test or Mann-Whitney's U test as appropriate, while qualitative variables were compared with the chi-square test or Fisher's exact test (if the expected cell frequencies were lower than 5). An ANOVA method with a general linear model (GLM) was used to evaluate overall comparisons. The primary endpoint (difference in the %IA of systemic arteries in COPD patients compared to smokers), and all other between-groups comparisons were performed using the least significant difference method with GLM. All adjusted analyses were performed with an ANCOVA method by GLM, using as covariates those variables associated with %IA in the overall population of the study ($p<0.10$): gender, MetS, cHDL and circulating leukocytes. Results are reported as least squares means (LSM) ± standard error of the mean (SEM) for the analyses detailed above. The linear regression analyses performed to determine which clinical factors were associated with the intimal thickening of systemic and pulmonary vessels are detailed in S1 File. Spearman's correlation coefficients (p) were used to assess the relationships between pairs of continuous intimal thickening parameters in systemic and pulmonary arteries. A two-tailed p-value of <0.05 was considered as statistically significant in all the tests performed. Statistical analysis was performed using PASW Statistics v18.0 software (SPSS Inc., Chicago, IL).

Results

Consecutive samples from 48 patients undergoing lung resection surgery were prospectively included in this protocol. However, six of them (12.5%) were excluded due to poor conservation of vessel architecture (n = 4) or incomplete measurable elastic laminas despite several attempts at the reorientation of the sample (n = 2).Therefore, 42 subjects with valid IC arteries were analysed. Seventeen in the group of COPD (current = 11 and former smokers = 6), 14 in the smokers group (current = 5 and former smokers = 9), and 11 in the non-smokers group. Overall, baseline variables were mostly well balanced between groups (data is summarized in Table 1). However, some differences were observed in the COPD group compared to smokers,

Table 1. Baseline demographics by groups.

Parameters	COPD (N = 17)	Smokers (N = 14)	Non smokers (N = 11)	Overall p-value
Male gender, n (%)	16 (94.1)†	13 (92.9)	4 (36.4)	<0.001
Age, years	63.4 [57.7–68.8]	58.3 [50.0–65.9]	66.1 [49.5–68.9]	0.524
BMI, kg/m^2	24.0 [21.7–27.7]	27.1 [25.0–30.4]	28.9 [21.5–30.1]	0.187
Pack-years	45 [39–60]†,‡	37.5 [20–41.3]	0 [0–0]	<0.001
Current smoking, n (%)	11 (64.7)†	5 (35.7)	0	0.003
Emphysema in CT, n (%)	12 (70.6)†,‡	4 (28.6)	0	0.001
Aortic calcifications, n (%)	13 (76.5)†	8 (57.1)	3 (27.3)	0.037
Systemic hypertension, n (%)	5 (29.4)	7 (50.0)	4 (36.4)	0.497
Diabetes Mellitus, n (%)	1 (5.9)‡	7 (50)	0	0.001
Renal failure, n (%)	0	0	0	-
Met.S, n (%)	6 (35.3)‡	11 (78.6)	3 (27.3)	0.016
Lipid lowering, n (%)	5 (29.4)	5 (35.7)	3 (27.3)	0.888
ACEIs/ARBs, n (%)	5 (29.4)	8 (57.1)	3 (27.3)	0.198
FEV_1 Post-BD, % predicted	62.0 [53.9–82.0]†,‡	97.7 [80.0–103.5]	107.0 [89.3–119.8]	<0.001
FEV_1/FVC Post-BD, %	54.9 [43.3–67.6]†,‡	76.3 [72.6–81.2]	77.2 [73.7–81.5]	<0.001
D_{LCO}, % predicted	68.3 [53.0–76.5]†,‡	87.1 [71.5–102.3]	84.4 [72.1–103]	0.001
LABA, n (%)	6 (35.3)‡	-	-	0.006
Inhaled CS, n (%)	5 (29.4)‡	-	-	0.015
WC, cm	83.1 [76.3–93.8]	90.9 [86.5–95.7]	88.4 [78.4–99.1]	0.299
SBP, mmHg	130 [120–137]	130 [121–138]	126 [120–135]	0.835
DBP, mmHg	73 [70–82]	74 [70–79]	70 [60–78]	0.340
cHDL, mmol/L	1.23 [0.90–1.37]†	1.19 [0.78–1.37]	1.55 [1.18–2.07]	0.030
cLDL, mmol/L	2.51 [1.91–3.19]	2.26 [1.23–3.05]	2.96 [2.24–3.50]	0.187
Total cholesterol, mmol/L	4.41 [3.68–5.03]	4.44 [3.70–4.99]	5.55 [3.88–5.86]	0.516
Triglycerides, mmol/L	1.25 [0.83–1.86]	1.99 [1.61–2.39]	1.60 [1.17–1.88]	0.131
FBG, mmol/L	5.6 [4.8–6.6]	6.7 [5.2–7.5]	5.4 [4.9–6.5]	0.074
Leukocytes count, x10E9/L	8.2 [7.7–9.9]†	7.8 [6.8–8.8]	6.3 [5.7–8.3]	0.045

Data are presented as median [25th-75th percentile]. BMI: body mass index, Met.S: Metabolic syndrome, ACEIs/ARBs: angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, FEV_1: forced expiratory volume in one second, BD: bronchodilator, FVC: forced vital capacity, D_{LCO}: diffusing capacity of the lungs for carbon monoxide, LABA: long acting β-agonists, CS: inhaled corticosteroids, WC: waist circumference; SBP: systolic blood pressure, DBP: diastolic blood pressure, cHDL: high-density lipoprotein cholesterol, cLDL: low-density lipoprotein cholesterol, FBG: fasting blood glucose.

† means a p-value <0.05 for the difference between COPD and non-smokers group, and

‡ means a p-value <0.05 for the difference between COPD and smokers group.

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where higher tobacco consumption (pack-years) was observed in COPD group, and a higher prevalence of metabolic syndrome and diabetes mellitus was seen in smokers. In the comparisons performed with the non-smokers group, predominance of male gender and the presence of aortic calcifications in the COPD and smokers groups were observed.

Morphometric measurements and severity indices of intimal thickening

Morphometric measurements of systemic arteries showed no differences in the dimensions of vessels or areas between groups (Table 2). The severity indices of intimal thickening as evaluated by non-adjusted analyses (%IA, %LN, ITI and IMR) were not significantly different between COPD and smokers. However, in the adjusted analysis %IA was significantly higher in COPD subjects compared to smokers. %LN and ITI showed a numerically increasing trend in the COPD group compared to the smokers group, while not attaining statistical significance.

Table 2. Morphometric measurements and severity indices of intimal thickening in systemic and pulmonary arteries by groups.

Measurements	Systemic arteries			
	COPD	Smokers	Non smokers	Overallp-value
Measured external diameter, μm	557 [485–668]	506 [440–599]	505 [427–632]	0.385
Measured total area, $mm^{-2}x10^{-3}$	239 [162–337]	199 [143–254]	189 [141–277]	0.338
Index of narrowing[a]	0.27 [0.24–0.33]	0.30 [0.23–0.34]	0.28 [0.27–0.32]	0.845
Lumen area, $mm^{-2}x10^{-3}$	49.9 [25.9–69.6]	47.4 [24.5–59.2]	58.8 [20.1–92.2]	0.811
Intima area, $mm^{-2}x10^{-3}$	32.0 [19.6–57.0]	26.8 [17.7–37.7]	23.4 [17.5–37.1]	0.327
Muscular area, $mm^{-2}x10^{-3}$	157.2 [115.5–201.9]	117.1 [96.4–161.1]	113.3 [72.9–164.8]	0.169
Severity Indices[b]				
% intimal area	15.6 ± 1.5†,‡	14.2 ± 1.6	13.1 ± 1.8	0.022
% luminal narrowing	44.9 ± 4.2†	40.2 ± 4.7	36.9 ± 5.3	0.030
Intimal thickness index	0.25 ± 0.03†	0.24 ± 0.03	0.21 ± 0.03	0.091
Intima to media ratio	0.46 ± 0.04	0.46 ± 0.05	0.37 ± 0.05	0.678
	Pulmonary arteries			
	COPD	Smokers	Non smokers	Overallp-value
N° of arteries measured by patient	10.2 ± 1.2	13.2 ± 1.8	8.7 ± 2.3	0.212
Measured external diameter, μm	294.2 [256.2–362.1]	323.9 [303.5–406.4]	327.5 [314.8–360.1]	0.176
Measured total area, $mm^{-2}x10^{-3}$	64.0 [49.8–99.1]	77.4 [64.2–121.6]	74.3 [65.8–84.7]	0.237
Index of narrowing[a]	0.30 [0.28–0.32]	0.31 [0.28–0.32]	0.30 [0.29–0.34]	0.862
Lumen area, $mm^{-2}x10^{-3}$	18.0 [12.9–23.4]‡	27.9 [25.3–37.3]	26.9 [16.9–35.4]	0.013
Intima area, $mm^{-2}x10^{-3}$	24.0 [18.2–32.7]	21.2 [16.4–33.3]	15.4 [12.2–21.1]	0.092
Muscular area, $mm^{-2}x10^{-3}$	21.8 [17.0–34.9]	27.5 [21.1–45.4]	30.3 [26.5–33.8]	0.291
Severity Indices[b]				
% intimal area	37.3 ± 2.2†,‡	29.3 ± 2.3	23.6 ± 2.8	0.002
% luminal narrowing	56.1 ± 3.1†,‡	43.4 ± 3.2	37.9 ± 4.1	0.003
Intimal thickness index	0.99 ± 0.06†	0.82 ± 0.06	0.54 ± 0.08	<0.001
Intima to media ratio	1.17 ± 0.10†	1.03 ± 0.11	0.80 ± 0.13	0.107

Data are presented as median [IQR] or LSM ± SEM. The reported p-value comes from the overall comparison with ANCOVA method with a general linear model using as covariables: gender, MetS, cHDL and circulating leukocytes.

Between-group comparisons were performed using the least significant difference method, where † means a p-value <0.05 for the difference between COPD and non-smokers group, and

‡ means a p-value <0.05 for the difference between COPD and smokers group.

[a]Index of narrowing is estimated as the ratio between the measured total area and that extrapolated from the theoretical distended diameter: [*theoretical diameter = length of the external elastic lamina / pi (π)*].

[b]The severity indices were calculated using the following formulas: % intimal area = 100 X intimal area/measured total area (area encompassed by the external elastic lamina); % luminal narrowing = 100 X intimal area/internal elastic lamina area; Intimal thickness index = intimal area/medial area; and Intima to media ratio = width of intima at maximal intimal thickness/width of media at maximal intimal thickness.

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Three of the four indices of intimal thickening (%IA, %LN and ITI) were significantly higher in the COPD group than in the group of non-smokers. Covariates included in the adjusted analyses were gender, MetS, cHDL and circulating leukocytes. Individual data and p-values of unadjusted and adjusted analysis of intimal thickening parameters by groups are represented in Fig 2A–2D. No severe lesions with calcification were observed in the arteries examined.

In muscular pulmonary arteries, the morphometric valuation revealed similar vessel dimensions among groups (Table 2). However, it was observed that two of the intimal thickening parameters (%IA and %LN) were significantly higher in the COPD group than in the smokers group. Furthermore, each of the 4 severity indices was significantly superior in the COPD

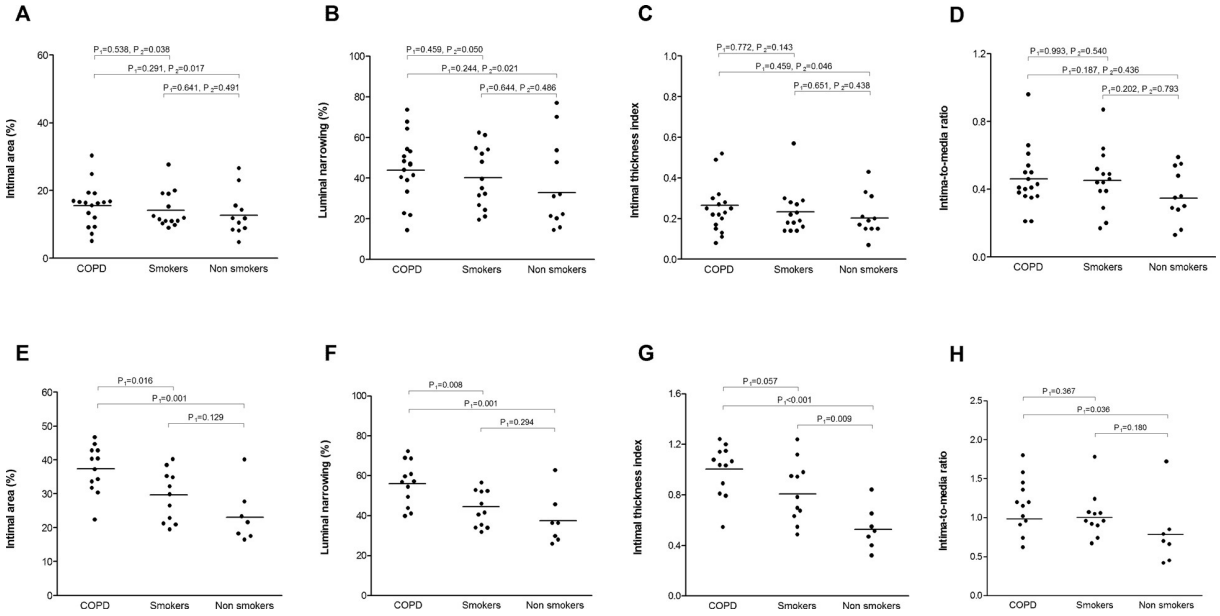

Fig 2. Severity indices of intimal thickening in systemic and pulmonary vessels, by groups. A-D represents individual data of systemic intimal thickening parameters: intimal area, luminal narrowing, intimal thickness index and intima-to-media ratio, respectively. **E-H** represents individual data of pulmonary intimal thickening parameters: intimal area, luminal narrowing, intimal thickness index and intima-to-media ratio, respectively. Horizontal bars indicate least squares mean values. P values for the pairwise comparisons using the least significant difference method with general linear model. P1: P-value for unadjusted analysis, P2: P-value for adjusted analysis using as covariables: gender, MetS, cHDL and circulating leukocytes.

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group compared to the non-smokers group. In addition, a greater degree of intimal thickening (ITI) was observed in the smokers group compared to the non-smokers group. It is noteworthy that the only clinical factor associated with the %IA of pulmonary vessels in the regression analysis was COPD status. Therefore, the data reported above are from the unadjusted analysis. Plots of individual data for intimal thickening of pulmonary arteries by groups are represented in Fig 2E–2H. There was agreement in morphometric measurements between the two observers with an intra-class correlation coefficient (ICC) of 0.907 (CI from 0.78 to 0.96, $p<0001$).

Factors associated with intimal thickening in systemic and pulmonary arteries

The results of univariate and multivariate regression analyses of the factors associated with intimal thickening in systemic arteries (%IA) are summarized in Table 3. Based on these results, five variables were identified ($p<0.10$) as possible risk factors: male gender, leukocytes count and cHDL (related to lower %IA) and MetS and COPD (related to higher %IA). In the analyses explained above, the presence of Diabetes Mellitus and the pack-years were not associated to % IA (Table 3). In the multivariate analysis, only MetS, gender and COPD were significantly associated with %IA. Table 4 shows the clinical variables tested as possible factors associated with pulmonary intimal thickening (%IA). Only COPD was significantly associated with %IA.

Correlations between systemic and pulmonary intimal thickening

Significant correlations were observed in the %IA between systemic and pulmonary arteries (Spearman's rho = 0.435, p = 0.016) in the overall population. However no correlation was observed between systemic and pulmonary arteries in %LN (Spearman's rho = 0.360, p = 0.051), ITI (Spearman's rho = 0.145, p = 0.445) and IMR (Spearman's rho = 0.061,

Table 3. Linear regression analyses for associations between clinical variables and the intimal thickening (%IA) of systemic arteries in the overall population.

Dependent variable:% Intimal area	Univariateβ coefficient [95% CI]	p-value	Multivariateβ coefficient [95% CI]	p-value
Male gender	-6.33 [11.20- -1.46]	0.013	-6.64 [-11.14- -2.14]	0.005
Age	0.061 [-0.14–0.26]	0.543		
BMI	0.15 [-0.35–0.64]	0.541		
Pack-years	-0.03 [-0.21–0.16]	0.758		
Emphysema	0.173 [-6.85–7.20]	0.960		
Systemic hypertension	-1.31 [-5.82–3.20]	0.555		
Diabetes Mellitus	0.86 [-6.13–7.84]	0.801		
Metabolic syndrome	5.78 [1.84–9.73]	0.006	5.52 [2.21–8.82]	0.002
Lipid lowering	1.48 [-4.03–6.98]	0.585		
ACEIs/ARBs	-0.20 [-8.65–8.25]	0.961		
cHDL	-3.59 [-7.79–0.60]	0.091	-3.32 [-7.10–0.45]	0.083
cLDL	0.62 [-1.81–3.14]	0.618		
Triglycerides	-1.24 [-4.71–2.24]	0.471		
Leukocytes	-0.89 [-1.94–0.16]	0.095	-0.64 [-1.55–0.27]	0.164
COPD	4.78 [0.53–9.03]	0.029	4.96 [1.43–8.48]	0.007

BMI: body mass index, ACEIs/ARBs: angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, cHDL: high-density lipoprotein cholesterol, cLDL: low-density lipoprotein cholesterol.

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p = 0.749). In Fig 3, microphotographs of systemic and muscular pulmonary arteries with ascending intimal thickening, belonging to the three groups of study are represented.

Discussion

The present investigation is, to the best of our knowledge, the first histo-morphometric analysis performed with the aim of evaluating concomitantly the intimal thickening of the systemic and

Table 4. Linear regression analyses for associations between clinical variables and the intimal thickening (%IA) of pulmonary arteries in the overall population.

Dependent variable:% Intimal area	Univariateβ coefficient [95% CI]	p-value
Male gender	-2.77 [-11.78–6.25]	0.530
Age	0.109 [-0.23–0.45]	0.515
BMI	-0.06 [-1.09–0.97]	0.902
Pack-years	0.09 [-0.04–0.23]	0.163
Emphysema	-1.79 [-9.83–6.24]	0.647
Systemic hypertension	-1.21 [-12.65–10.23]	0.827
Diabetes Mellitus	-4.61 [-12.22–2.99]	0.223
Metabolic syndrome	0.94 [-9.01–10.89]	0.844
Lipid lowering	3.37 [-2.47–9.20]	0.247
ACEIs/ARBs	1.52 [-8.16–11.19]	0.746
Inhaled CS	4.32 [-5.91–14.55]	0.391
Leukocytes	-0.86 [-2.38–0.65]	0.252
COPD	10.23 [4.37–16.09]	0.001

BMI: body mass index, ACEIs/ARBs: angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, CS: corticosteroids.

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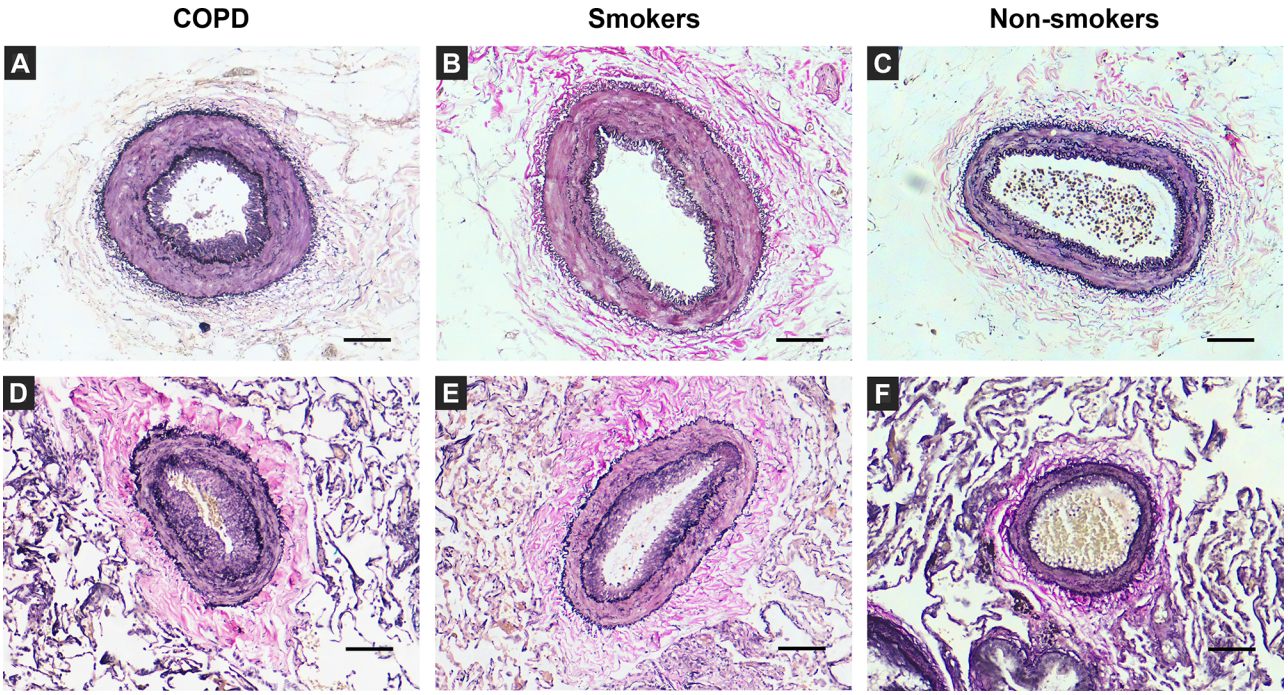

Fig 3. Representative photomicrographs of orcein stained elastin fiber in cross sections of intercostal and muscular pulmonary arteries. Images from A to C represent intercostal arteries sections. The intima in **A** (COPD) is thicker than in the smokers (**B**) and the non-smokers (**C**) group. Images from D to F show the muscular pulmonary arteries sections of COPD (**D**), smokers (**E**), and non-smokers (**F**). Vessel remodelling, characterized by thickening of the intima layer, is more evident in the COPD group. Elastin-orcein stain, scale bar = 100μm.

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pulmonary arteries of COPD subjects compared to smokers. The principal findings of this study can be summarized as follows: 1) Patients with COPD have an increased %IA of systemic and pulmonary arteries compared to smokers with normal lung function; 2) %IA of systemic arteries is independently related to gender, the presence of metabolic syndrome and COPD, while the %IA of pulmonary arteries is related only with COPD; 3) there is a correlation between the %IA of systemic and pulmonary arteries in the overall population.

Previous studies have shown that the intimal thickening of systemic arteries is due to the increased formation of extracellular matrix (ECM), which includes an increased secretion and deposition of proteins, growth factors and enzymes that regulate ECM.[14] These remodeling changes could contribute to different clinical vascular disorders such as multifactorial atherosclerosis.[15] In the context of the current study, four methods were used to evaluate the propensity of in situ IC artery and muscular pulmonary arteries to develop intimal thickening: % IA, %LN, ITI and IMR. The %IA, %LN and ITI indices are measures of the severity of intimal thickening that allow for an accurate evaluation of eccentric or irregular disease since they consider areas rather than widths. Also, they could be useful for comparing intimal thickening between different vascular beds.[12] IMR is an alternative method for comparing intimal disease, and it specifically evaluates the severity of atherosclerosis, in which the maximal thickness of intima from cross sectional is considered and is presumed to be a useful parameter for comparisons of the same artery among different patients but cannot be used to compare different vascular beds.[12] In the present study, it was found that COPD subjects exhibit an increased intimal thickening of systemic arteries in terms of %IA and a numerically increasing trend in terms of %LN and ITI compared to smokers; all three of these parameters are area-dependent. Nonetheless, no differences were found in IMR, which could be explained by the irregular

intimal thickening observed. Overall, these results support the idea that COPD patients exhibit a major intimal hyperplasia in systemic circulation compared to smokers with normal lung function. This novel data is of particular relevance since these vascular changes could be the initial lesions in a complex process resulting in atherosclerosis or the beginning of a non-atherosclerosis related subclinical vascular disease.[16] In line with this, prior studies using different non-invasive measurements to evaluate subclinical vascular disease determined an increased cardiovascular risk in stable COPD subjects independent of age or smoking status.[17–20] Specifically, the ultrasonographic evaluation of vascular remodelling showed that carotid artery wall thickening (carotid intima-media thickness) was associated with the severity of airflow limitation in COPD subjects.[17–20] Similarly, there are data supporting the association of more advanced vascular disease in elderly subjects with COPD, assessed by high-resolution magnetic resonance imaging, where COPD was an independent predictor for the presence of a lipid core, and therefore of vulnerable plaque in the carotid artery.[17] Of note, the current analysis of IC arteries of the non-smoker and smoker group show a similar intimal layer as in morphometric studies in post-mortem subjects who had died of non-cardiac diseases,[21] which gives consistence to our results.

Most of the studies focussing on atherosclerosis have been performed in arterial tissue from arterectomies or are post-mortem studies carried out in advanced stages of the disease.[21] However, a valid alternative to studying the initial processes is the evaluation of the 5th IC artery which arises from the descending thoracic aorta and provides blood supply to the ribs, IC spaces and skin of the anterolateral thoracic wall.[22] Of all IC arteries, the 5th IC artery has the greatest luminal diameter, length and rate of flow,[23] which makes it accessible in all patients undergoing open thoracotomy. Also, of the three segments of the IC artery: proximal, middle and distal, the middle level (analyzed in this study), has a thin inner layer and a muscular or elasto-muscular media layer, characterized by an absence or by few elastic laminas except for the IEL and EEL, respectively.[21] These two features result in a greater tendency toward intimal hyperplasia as compared to elastic conduits,[13,15] therefore muscular IC artery appears histologically more in the coronary arteries or radial arteries, where remodelling is most evident.[15,21,23]

Risk factors for the intimal thickening of systemic arteries vary widely between morphometric studies; in general the clinical variables tested are the same ones that are related to clinical atherosclerosis. However, previous studies have demonstrated that different arterial beds have different risk factors for the development of intimal hyperplasia and atherosclerosis.[13] The most common clinical factors associated with intimal hyperplasia in different arteries (non IC artery), as measured by different methods are: diabetes mellitus, renal failure, hypertension, peripheral vascular disease, smoking and age.[13] In our study, the factors associated with the intimal thickening of the IC artery as measured by %IA were female gender, metabolic syndrome and COPD status. Although female gender may have different features in the functional and morphometric behavior of systemic arteries, as previously reported;[24] in our study, the poor representation of women in some of the groups analyzed makes the interpretation of results difficult, as we discussed in the limitations section. In pulmonary circulation, the results of an increased intimal thickening of the muscular pulmonary arteries of COPD subjects are in agreement with prior morphometric studies that have demonstrated a higher proliferation of smooth muscles cells and the deposition of ECM proteins in the intima layer of the pulmonary vessels in COPD subjects.[7,25]

Another novel issue in this study is the relationship between systemic and pulmonary vascular remodeling changes assessed by histo-morphometric analysis. Previous studies evaluating pulmonary vascular disease (measured by the cross sectional area of small pulmonary vessels in computerized tomography) or histological pulmonary atherosclerosis (in post-mortem

analysis) showed a positive correlation with the atherosclerosis of systemic arteries.[26–27] Although the underlying mechanisms are still unknown, it is hypothesized, that systemic inflammation and endothelial dysfunction may promote both systemic and pulmonary vascular alterations, and may lie behind the close relationship between both conditions.[28] As previously reported in systemic sclerosis, an immunologic disorder with a simultaneous impairment of systemic and pulmonary circulation could occur.[29]

Limitations

The principal limitation of the study is the poor representation of female gender in the COPD and smokers groups due to the baseline characteristics of our population (patients with lung carcinoma and a major smoking habit are mostly male patients). Therefore, this gender misbalance makes it difficult to draw conclusions about gender beyond spurious associations. The population of the study has primary, treatable lung cancer; therefore lung cancer could be a possible introduced bias. However, we are assuming any effect would be the same across all the subjects included in the study. It is important to consider that it would be impossible to obtain lung tissue and arterial samples from living patients, if it were not for the indication of the surgery. Another possible limitation of the study is that, due to its observational design, causal conclusions or strong conclusions cannot be drawn beyond observing a significant association between the presence of COPD and vascular remodelling changes in systemic and pulmonary arteries.

Conclusions

In conclusion, the current study shows a greater intimal thickening in systemic and pulmonary arteries in the COPD group compared to smokers group. These histopathological observations imply that subjects with a mild-moderate COPD in addition to pulmonary vascular involvement, also show systemic vascular changes (histological remodelling) which could explain the high prevalence of cardiovascular disease in COPD patients. Therefore, in clinical practice, the adequate management of pulmonary disease and others modifiable cardiovascular risk factors should be a target in all subjects with COPD, even in mild stages, in order to minimize cardiovascular consequences. The present study provides a model for future studies involving initial remodelling changes in both systemic and pulmonary circulations.

Supporting Information

S1 File. Additional definitions and details of analyses.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MME SS MLS DH IE RP MM FM JD. Performed the experiments: MME MLS MM RP IE SS. Analyzed the data: MME MLS DH MM SS RP. Contributed reagents/materials/analysis tools: MME MM JD FM RP IE. Wrote the paper: MME MLS IE DH RP MM FM JD SS.

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Estudio II. Vascular Disease in COPD: Systemic and Pulmonary expression of PARC (Pulmonary and Activation-Regulated Chemokine)

Muñoz-Esquerre M, Aliagas E, López-Sánchez M, Escobar I, Huertas D, Penín R, Dorca J, Santos S.

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RESEARCH ARTICLE

Vascular disease in COPD: Systemic and pulmonary expression of PARC (Pulmonary and Activation-Regulated Chemokine)

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Abstract

Introduction

The role of Pulmonary and Activation-Regulated Chemokine (PARC) in the physiopathology of Chronic Obstructive Pulmonary Disease (COPD) is not fully understood. The aim of the present study is to analyze the expression of PARC in lung tissue and its relationship with the vascular remodeling of the systemic and pulmonary arteries of COPD subjects.

Methods

To achieve this objective, protein and gene expression experiments, together with ELISA assays, were performed on the lung tissue, intercostal arteries and serum samples from COPD patients, non-obstructed smokers (NOS) and never-smokers (NS).

Results

A total of 57 subjects were included in the analysis (23 COPD, 18 NOS and 16 NS). In the comparisons between groups, a significantly increased lung protein expression of PARC was observed in the COPD group compared to the NOS group (1.96±0.22 vs. 1.29±0.27, P-adjusted = 0.038). PARC was located predominantly in the smooth muscle cells of the remodeled pulmonary muscular arteries and the macrophage-rich area of the alveolar parenchyma. No differences were detected in PARC gene expression analyses. The protein content of PARC in the intercostal arteries were similar between groups, though little remodeling was observed in these arteries. Circulating levels of PARC were numerically higher in patients with COPD compared to NOS and NS.

Conclusion

The results of the present study suggest an increased lung protein expression of PARC in COPD subjects. This protein was mainly localized in the smooth muscle cells of the

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pulmonary muscular arteries and was associated with the severity of intimal thickening, indicating its possible role in this remodeling process.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is characterized by an abnormal inflammatory response of the lungs to noxious particles or gases, particularly cigarette smoke [1–2].Cardiovascular disease (CVD) is the most important comorbidity associated with COPD, due to its impact on patients' overall prognosis, including mortality [1,3–5].It has been suggested that this systemic inflammation may be one of the main factors that play a significant role in the pathogenesis of atherothrombosis in COPD [6–7]. In this setting, chemokines are a group of chemotactic molecules that appear to regulate the directed movement of leukocytes and may therefore play important roles in inflammation and immunity [8].Of particular interest is the Pulmonary and Activation-Regulated Chemokine (PARC), also known as CCL18 or Macrophage Inflammatory Protein-4 (MIP-4), which is a new member of the CC chemokine family [8]. Although early studies described the constitutive lung tissue expression of PARC in humans [8–9], the role of PARC in the physiopathology of COPD and its relationship with the systemic vascular involvement described in this chronic condition are currently unknown [10].A small number of studies suggest that PARC could be a serum biomarker of cardiovascular mortality in large populations of COPD patients [11–12]. However, to the best of our knowledge, there are no previous data directly addressing the tissue characterization of PARC in COPD. Therefore, the current hypothesis was that PARC expression could be modified in COPD. The aim of the present study was to analyze the expression of PARC at the pulmonary, systemic and circulatory levels in the context of this respiratory disease. To achieve this objective, protein and gene expression experiments, together with ELISA assays, were performed on lung, intercostal (IC) artery and serum samples from COPD patients, non-obstructed smokers (NOS) and never-smokers (NS).The correlation between the immunostaining of PARC in both tissues (lung and systemic arteries) and their intimal thickening were studied.

Materials and methods

Subjects

This was a prospective study, performed in consecutive subjects who underwent lung resection (lobectomy or pneumonectomy) for the treatment of localized primary lung cancer. In line with the current definition of COPD in the GOLD guidelines [1],patients were divided into three groups: 1) COPD subjects (all of them current or former smokers), 2) non-obstructed smokers (NOS), and 3) never-smokers (NS). All procedures were perform in accordance with the Declaration of Helsinki, and protocols were approved by the local ethics committee "Comitè Ètic d' Investigació Clínica del Hospital de Bellvitge, N° PR006/11", An informed consent form was obtained from all participants.

Sample collection

Lung specimens, sections of the 5th posterior IC artery and venous blood samples, were collected from all subjects. All lung tissue samples were obtained at a minimum distance of 5 cm from the tumor localization. Tissues samples were fixed overnight in 4% paraformaldehyde and embedded in paraffin. A microscopic evaluation was performed on the lung tissue to

confirm the absence of neoplastic cells before it was included in the analysis. Venous blood samples were collected prior to surgery. Serum was extracted and stored at -80˚C until it was used.

Western blot assays

The PARC protein expression of lung and IC artery was examined by western blot analysis. Protein concentrations were measured using the Lowry method. In short, 40μg of lung and 50μg of IC artery protein homogenates were loaded to pre-cast 4–20% polyacrylamide-SDS gradient gel for electrophoresis and then transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in tris buffer saline containing 0.1% Tween® 20 (TBS-T) and 5% bovine serum albumin (pH 7.4) for 1.5 hours (h) at room temperature (RT). The primary antibody against PARC was incubated at RT for 1hour (1/1000, AB104867, Abcam, Cambridge, UK). After 3 washes with TBS-T, membranes were incubated for 1h with polyclonal goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1/2000; Dako, Carpinteria, CA, USA). The Clarity Western ECL System (Bio-Rad) was used to detect the protein signal. Results were digitized using the Image Reader LAS-3000 (Fujifilm, Tokyo, Japan). Band density was quantified by densitometry using Multi-gauge v1.3 software and normalized to β-actin levels (AB8226, Abcam).

Immunolabeling experiments

Immunohistochemistry and immunofluorescence experiments were carried out in order to study the expression and the exact localization of PARC in both tissues (lung and systemic arteries). Briefly, paraformaldehyde fixed paraffin embedded tissue sections of 4 μm were deparaffinised, rehydrated and rinsed in phosphate buffer saline (PBS). Antigen retrieval was for 1 minute at 100˚C using a citrate buffer, pH 6. After 3 rinses in PBS, tissue sections were pre-incubated for 2 hours at RT in 20% normal goat serum (Gibco, Paisley, UK), 0.2% gelatin (Merck, Darmstadt, Germany) and 0.1% triton® X-100 (Sigma-Aldrich, Sant Louis, Missouri, MO, USA). Slices were then incubated overnight at 4˚C with the following primary antibodies: rabbit anti-human MIP-4 (1/400, Peprotech, Rocky Hill, New Jersey, NJ, USA), and in fluorescence experiments, rabbit anti-human MIP-4 (1/250, Abcam) or mouse anti-human alpha smooth muscle actin (αSMA) clone 1A4 as a smooth muscle cells (SMC) marker (1/400, A 5228, Sigma). After three washes in PBS-triton, samples were incubated with the avidin-biotin complex/peroxidase (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) or, in fluorescent assays, with Alexa Fluor 488- or 555-goat anti-mouse or anti-rabbit (Life technologies, Paisley, UK) for 1 h at RT. Nuclei were counterstained with haematoxylin or, alternatively, in fluorescent assays, To-Pro®-3 (Life technologies) were used to visualize the nuclei. The results were observed and photographed under Leica DMD 108 light microscope (Leica Microsystems, Wetzlar, Germany) or, in fluorescence assays, under a Leica TCS-SL spectral confocal microscope (Leica).

Immunohistochemistry evaluation was performed in all samples and was used to semi-quantify the PARC expression in both tissues. Two blind observers performed the analysis of the following structures: 1) muscular pulmonary arteries (external diameter between 100 and 500 micrometers), 2) alveolar parenchyma, 3) bronchus and 4) IC arteries. In both pulmonary muscular and IC arteries, the labeling of the intima, medial and adventitia layers was assessed. The bronchial evaluation included epithelial cell layer, sub-epithelial baseline membrane (SEBM), and airway SMCs. Label intensity was scored as negative (0), mild (1), moderate (2), or strongly positive (3). The percentage of positive structures and the average score were computed for pulmonary muscular arteries and bronchial structures in each subject. Immunofluorescence experiments were performed to confirm

the exact localization of PARC protein in pulmonary and intercostal arteries with a high PARC expression previously observed by immunohistochemistry experiments.

Vascular morphometry

The histological and morphometric characteristics of both pulmonary muscular arteries and intercostal arteries were analyzed as described in detailed previously [10], following stereological methods for sampling and fixation for vascular structure evaluation [13]. In brief, tissue was stained with haematoxylin & eosin and orcein to differentiate the internal elastic lamina (IEL) and external elastic lamina (EEL). Vessel cross-sections of intercostals arteries and only pulmonary muscular arteries with an external diameter of 100 to 500 micras were considered in the analysis. Using a computerized image analyzer, the areas occupied by the lumen, the intima and the muscular layer were expressed as a percentage of the total area encompassed by the EEL. The degree of intimal thickening was defined by the percentage of intimal area (% IA = 100X intimal area/ measured total area or area encompassed by the EEL).

Quantitative real-time-PCR

Quantitative real-time-PCR (qRT-PCR) was performed to determine the gene expression of PARC in lung and IC artery tissue samples. Total RNA was isolated using trizol reagent (Life technologies). Genomic DNA digestion and RNA purification was performed with the DNasa I amplification grade kit (Life technologies). Total purified RNA (1 μg) was reversely transcribed into complementary DNA (cDNA) using the High capacity cDNA kit with RNAse inhibitor (Applied Biosystems, Foster City, CA, USA). Onemicroliter of cDNA was used to perform qRT-PCR using commercial inventoried Taqman assays for PARC (Applied Biosystems Taqman Assay, Hs00268113_m1).qRT-PCR reactions were carried out using the ABI Prism 7900HT Real Time PCR System (Applied Biosystems). Data were collected using SDS software v2.4 (Applied Biosystems) and analyzed by the comparative Ct (ΔΔCt) quantification method using Expression Suite v1.0.3 software (Applied Biosystems). The relative expression of PARC was determined using 18S mRNA (Taqman Assay, Hs03928985_g, Applied Biosystems) as an endogenous control. A common calibrator for each plate was used. Data are reported as a fold change ratio (RQ) of mRNA of PARC from each tissue sample.

Enzyme-linked immunosorbent assay

To determine circulating levels of PARC in all subjects; an ELISA kit was employed (AB100620-MIP4, Abcam) following the manufacturer's instructions. Samples were assayed in duplicate. A400 fold serum dilution was used for all samples. The sensitivity of the test was 2pg/ml.

Study endpoints and sample size calculation

The primary endpoint of this study was the difference in the lung protein content of PARC in patients in the COPD group compared to those in the NOS group. Assuming a standard deviation of 0.22 in band density, a sample size of 16 subjects per group was needed to detect a minimal difference of 0.20 between groups; with 80% power and a two-tailed p-value less than 0.05. Considering an approximate 20% dropout rate (e.g. inadequate samples for measurements), the inclusion of 20 subjects per group was allowed to ensure that data from 16 patients was available for analysis. Secondary endpoints included between-groups comparisons for mRNA expression and immunoreactions for PARC in pulmonary and systemic arteries and their circulating levels of PARC.

Statistical analysis

For baseline characteristics, continuous variables were expressed as mean ± SD or median and interquartile range whether a normal distribution was assumed or not (Kolmogorov-Smirnov test), respectively. Comparisons of continuous variables were performed with the analysis of variance (ANOVA) method or Kruskal-Wallis test as appropriate, while qualitative variables were compared with the chi-square test or Fisher's exact test (any expected value <5). An ANOVA method with a general linear model was used to evaluate the primary endpoint and all other between-group comparisons. Adjusted analyses were performed with an ANCOVA method, using unbalanced demographic variables as covariate (gender, pack-years and the presence of diabetes mellitus, $P < 0.05$). Spearman's correlation coefficients were used to assess the relationship between the percentage of intimal area and PARC immunostaning (labeling intensity score) in pulmonary and IC arteries. A two-tailed P value of <0.05 was considered to indicate a statistically significant difference. Results are reported as least squares mean (LSM) ± standard error of the mean (SEM) for the above detailed analyses. Statistical analysis was performed using PASW Statistics v18.0 software (SPSS Inc., Chicago, IL, USA).

Results

Consecutive samples from 63 patients undergoing lung resection surgery were included in the study, though six were discarded due to the poor quality or insufficiency of the sample obtained. Therefore, 57 patients were included in the present analysis, 23 COPD subjects, 18 NOS and 16 NS. There were no significant differences in baseline characteristics (Table 1) between groups, except for gender, tobacco exposure and the presence of diabetes; these were therefore included as covariables in all adjusted analyses.

Table 1. Baseline characteristics.

Parameters	COPD (N = 23)	NOS (N = 18)	NS (N = 16)	Overall P-value
Male gender, n (%)	21 (91.3)	17 (94.4)	6 (37.5)	<0.001
Age, years	64.4 [60.8–68.8]	61.0 [51.8–68.9]	65.7 [48.0–68.8]	0.75
BMI, kg/m^2	25.4 [21.8–28.3]	27.1 [24.9–29.9]	26.9 [23.6–30.1]	0.483
Pack-years	42 [35–60]	38 [20–41]	0	<0.001
Systemic hypertension, n (%)	10 (43.5)	8 (44.4)	4 (25)	0.346
Current smokers, n (%)	17 (73.9)	8 (44.4)	0	0.055
Diabetes Mellitus, n (%)	7 (30.4)	8 (44.4)	1 (6.3)	0.044
FEV_1 Post-BD, % predicted	62.7 [56.1–76.9]	96.7 [85.3–103.6]	103.2 [88.5–118.8]	<0.001
FEV_1/FVC Post-BD, %	59 [48.4–67]	74.9 [71.4–81.2]	78.2 [74.1–81.9]	<0.001
D_{LCO}, % predicted	70.9 [56.8–79.6]	81 [71.5–102.3]	92.2 [76.7–102.3]	0.002
LABA or LAMA, n (%)	13 (56.5)	0 (0)	0 (0)	<0.001
Inhaled CS, n (%)	7 (30.4)	0 (0)	0 (0)	0.003
Leukocytes count, x10E9/L	8.6 [7.6–10]	8.1 [6.9–9.1]	6.4 [5.6–8.3]	0.004
C-reactive protein, mg/L	3.5 [1.4–9.5]	2.4 [1.0–10.1]	1.2 [1.0–2.7]	0.240
Fibrinogen, g/L	3.2 [2.7–3.4]	3.1 [2.6–3.8]	2.8 [2.3–3.7]	0.696

Data are presented as median [25th-75th percentile]. The reported p-value comes from the overall comparison with ANOVA method or Kruskal-Wallis test as appropriate, while qualitative variables were compared with chi-square test or Fisher's exact test (any expected value <5). COPD: Chronic Obstructive Pulmonary Disease, NOS: non-obstructed smokers, NS: never-smokers, BMI: body mass index, FEV_1: forced expiratory volume in one second, BD: bronchodilator, FVC: forced vital capacity, DLCO: diffusing capacity of the lungs for carbon monoxide, LABA: long acting β-agonists, CS: corticosteroids.

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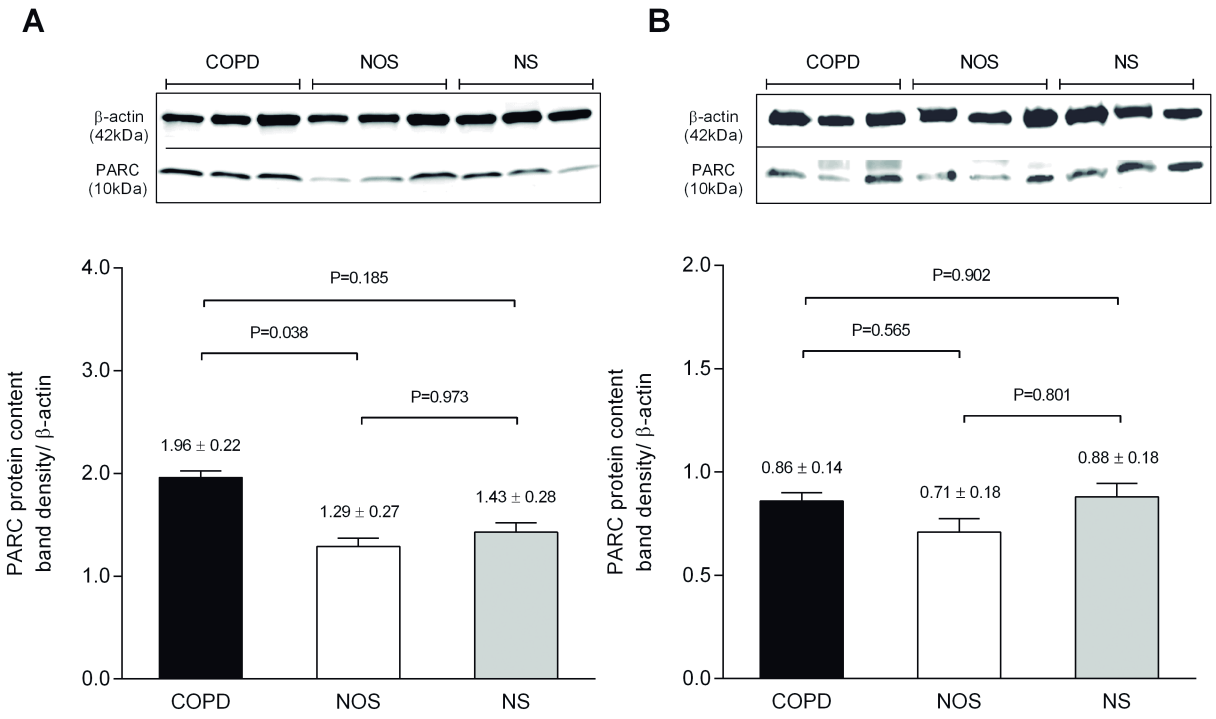

Fig 1. Western blot analysis for PARC. Upper panel: representative membranes of the Western blotting in homogenized lung tissue (A) and intercostal arteries (B). Bands at 37kD and 10kD are consistent with the size of β-actin and PARC respectively. Lower panel: (A) Band density analysis of 33 lung samples (12 COPD, 11 NOS and 10 NS). Of note, protein content was significantly increased in the COPD group compared to NOS. (B) Band density analysis of 28 intercostal artery samples (12 COPD, 8 NOS and 8 NS). There were no differences in PARC content between groups. Data are presented as LSM ± SEM. The reported p-value comes from the pair wise comparison with a general linear model using as covariables: gender, pack-years and the presence of diabetes mellitus. COPD: Chronic Obstructive Pulmonary Disease; NOS: non-obstructed smokers; NS: never-smokers.

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Protein expression analysis by western blot

Total PARC content in lung and IC artery was measured by western blot. In the lung, protein band density was significantly increased in the COPD group compared to NOS (1.96 ± 0.22 vs. 1.29 ± 0.27, P-adjusted = 0.038). No differences were observed in other between-group comparisons (Fig 1). In the case of IC arteries, PARC content was similar in all groups as shown by the densitometric analysis of the bands (Fig 1).

Protein expression and cellular localization of PARC

In order to further characterize the protein expression of PARC in lung and IC artery tissue samples, we carried out immunohistochemistry experiments (the results of which are summarized in Table 2). In the lung, PARC was predominantly immunolocalized in the SMC layer of the pulmonary muscular arteries and in the alveolar parenchyma (mostly in macrophage-rich areas), with a mild expression in bronchial structures (Fig 2). In the comparisons between groups, the percentage of positive pulmonary arteries with strong immunostaining at the muscular layer was higher in the COPD group compared to other groups (Table 2). In IC arteries, PARC was also found to be expressed predominantly in SMC, thoughno labeling differences were observed between groups (Fig 2). Notably, inflammatory cells were not observed in any

Table 2. Protein expression of PARC in lung tissue and intercostal arteries, according to groups.

Tissue	Parameters	COPD	NOS	NS	Overall P-value
Lung					
	Number of arteries measured in each subject	10.2±1.6	12.4±1.6	9.9±1.9	0.365
	% Positive immunoreaction	72.3 ± 8.1	57.6 ± 8.1	42.9 ± 9	0.063
	Endothelial layer *	0.02 ± 0.03	0.07 ± 0.03	0.08 ± 0.03	0.443
	Intimal thickening *	0.20 ± 0.07	0.13 ± 0.07	0.16 ± 0.08	0.772
	Muscular layer *	1.23 ± 0.15	0.86 ± 0.15	0.61 ± 0.16	0.023
	Adventitial layer*	0.15 ± 0.08	0.1 ± 0.08	0.05 ± 0.09	0.68
	Alveolar parenchyma*	1.33 ± 0.13	1.13 ± 0.13	0.83 ± 0.15	0.059
	Bronchial structures				
	% Positive immunoreaction	79.9 ± 9	59.9 ± 8.7	68.7 ± 9.4	0.293
	Bronchial epithelium*	0.59 ± 0.12	0.41 ± 0.12	0.6 ± 0.12	0.441
	SEBM*	0.1 ± 0.06	0.05 ± 0.06	0.03 ± 0.06	0.7
	Connective tissue*	0.49 ± 0.14	0.53 ± 0.14	0.3 ± 0.15	0.511
	ASM*	0.1 ± 0.08	0.21 ± 0.08	0.03 ± 0.08	0.242
Intercostal artery					
	Endothelial layer*	0.38 ± 0.2	0.31 ± 0.23	0.33 ± 0.24	0.975
	Intimal thickening*	0.31 ± 0.16	0.15 ± 0.18	0.42 ± 0.19	0.597
	Muscular layer*	1.19 ± 0.23	1.08 ± 0.26	1.58 ± 0.27	0.362
	Adventitial layer*	0.56 ± 0.17	0.54 ± 0.19	0.75 ± 0.2	0.694

Data are presented as LSM ± SEM. The reported p-value comes from the overall comparison with ANCOVA method with a general linear model using as covariables: gender, pack-years and the presence of diabetes mellitus.

*Label intensity was scored as negative (0), mild (1), moderate (2), and strongly positive (3). The percentage of positive structures and the average score were computed for pulmonary muscular arteries and bronchial structures in each subject. COPD: Chronic Obstructive Pulmonary Disease; NOS: non-obstructed smokers; NS: never-smokers.

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layer of the IC arteries evaluated by immunohistochemistry. Additionally, the presence of PARC in the SMC layer of both pulmonary and IC arteries was confirmed by double immunostaining performed with anti-MIP-4 and anti-αSMA antibodies. A partial tissue colocalization between PARC and α-SMA is shown in Fig 3.

Relationship between the vascular morphometry and the immunohistochemical expression of PARC

According to morphometric analysis, the percentage of the intimal area of pulmonary muscular arteries was significantly increased in the COPD group compared with the NOS and NS groups. However, in the case of the IC arteries, this intimal thickening shows a numerically increasing trend in the COPD group compared to the NOS and NS groups, though it did not attain statistical significance. In both types of arteries, there were no differences in the thickness of the muscular layer between groups. Data relating to morphometric measurements are shown in Table 3. In addition, representative microphotographies of pulmonary and intercostal intimal thickening are shown in Fig 4. In the pulmonary arteries, a significant correlation between the percentage of intimal area and PARC expression in the muscular layer was found (Spearman's rho = 0.39, p = 0.032) but not in the intimal layer. In the IC arteries no correlations between intimal thickening and the protein expression of PARC were detected (data not shown).

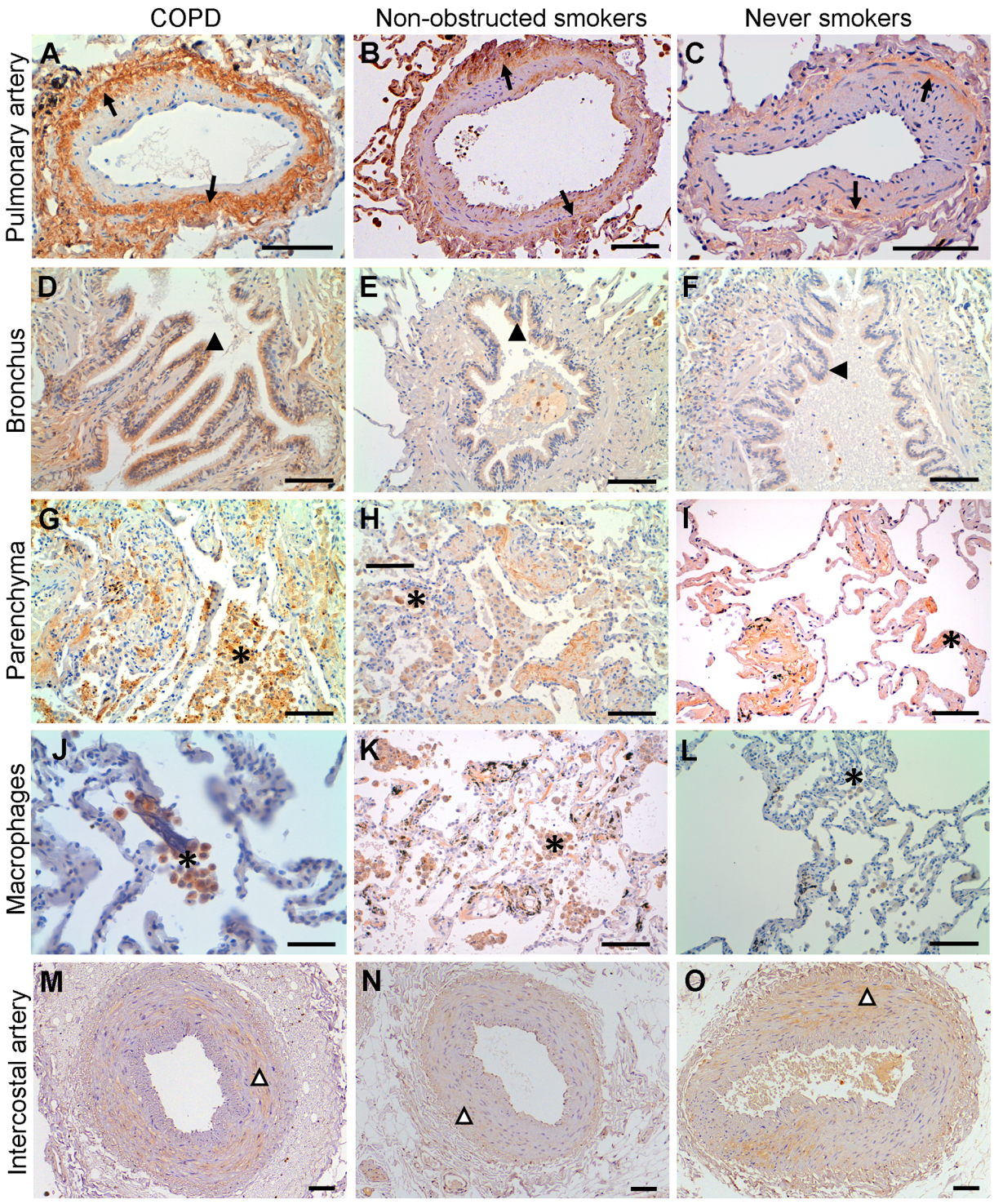

Fig 2. Immunolocalization of MIP-4/PARC. MIP-4/PARC was immunodetected in pulmonary muscular arteries (A-C), bronchus (D-F), alveolar parenchyma (G-I) and macrophages (J-L) as well in the intercostal arteries (M-O) of all biological groups. In pulmonary muscular arteries, MIP-4/PARC was mainly expressed in the muscular layer (arrows). Note that MIP-4/PARC expression is stronger in COPD. In bronchus, MIP-4/PARC was immunolocalized in epithelial cells (filled arrowheads) and its expression was similarly in all biological groups. The immunoreactivity of MIP-4/PARC was higher in the parenchyma of COPD patients. It is show in the magnification images of parenchyma (J-L), labeled macrophage rich areas (asterisks). In the intercostal arteries, MIP-4/PARC expression was mainly localized in the muscular layer (empty arrowheads) but no expression differences between groups were found. Images are representative histological slides from n = 23 COPD, 18 non-obstructed smokers and 16 never smokers. Scale bars = 100 μm except for images in (J-L), where they are 50 μm.

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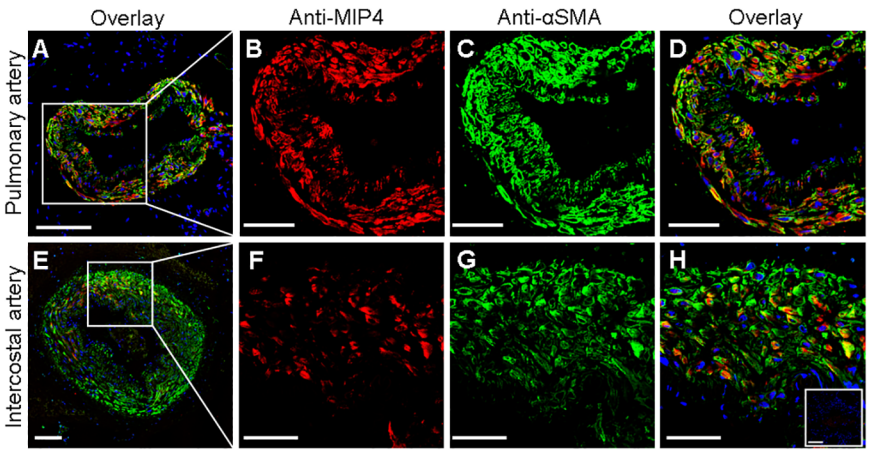

Fig 3. Colocalization of MIP-4/PARC and alpha smooth muscle actin (αSMA) in arterial tissue. Representative confocal fluorescence images of human pulmonary muscular (A) and intercostal (E) arteries labeled with antibodies against MIP-4/PARC (red) and αSMA (green). Nuclei were stained in blue. Higher-magnification (x40 oil lents) of representative images shows that MIP-4/PARC (B and F) and αSMA (C and G) were predominantly localized in the media layer of pulmonary and intercostal arteries. Overlay images show, in yellow, a partially colocalization between MIP-4/PARC and αSMA (D and H). Inset in H corresponds to control experiments performed with secondary antibodies alone. Scale bars = 50 μm except for images A and E, where they are 100 μm.

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Gene expression in lung tissue and intercostal artery tissue

No differences in the gene expression for PARC measured by the fold change of mRNA were observed between groups in lung tissue or in systemic arterial tissue in the analysis. In the overall population, the mRNA for PARC was constitutively expressed in lung samples (mRNA fold change of 1.02±0.18). However in the intercostal arteries, the expression was low compared to the endogenous control gene 18S (mRNA fold change of 0.48±0.12). Analysis of gene expression by groups is shown in Fig 5.

Table 3. Morphometric parameters of pulmonary muscular arteries and intercostal arteries.

	COPD (N = 23)	NOS (N = 18)	NS (N = 16)	Overall P-value
Pulmonary muscular arteries				
Total area, $mm^{-2}x10^{-3}$	64 [49.8–99.1]	82.7 [64.9–117.4]	75 [67.7–83.4]	0.174
Diameter, μm	294.2 [256.2–362.1]	329.1 [305.7–404.1]	330.6 [317.6–356.1]	0.15
Lumen area %*	25.3 [20.6–34.5]	34.8 [27.4–39.9]	40.2 [29–43]	0.045
Intimal area %*	38.8 [32.2–42.8]	30.5 [21.6–35.1]	22.5 [17.7–37]	0.02
Muscular area %*	33.9 [31.7–38.7]	36.5 [29.6–41.6]	38.2 [34.6–42]	0.519
Intercostal arteries				
Total area, $mm^{-2}x10^{-3}$	239.2 [161.7–336.8]	214.6 [145.6–270.2]	200 [142.3–273.5]	0.483
Diameter, μm	556.8 [485.4–668.4]	519.8 [440.1–612.4]	514. [429.6–624.1]	0.515
Lumen area %*	18.7 [15–23.7]	21.3 [12.9–28.3]	26.6 [12.4–34.3]	0.518
Intimal area %*	16.4 [10.6–18.1]	11.9 [11–19.1]	11.8 [8.6–21.2]	0.547
Muscular area %*	65 [59.9–70.7]	64.3 [48.8–68.8]	61 [50–69.3]	0.372

Data are presented as median [25th-75th percentile]. The reported p-value comes from the overall comparison with Kruskal-Wallis test.
*The areas occupied by the lumen, the intima and muscular layer were expressed as a percentage of the total area encompassed by the external elastic lamina. COPD: Chronic Obstructive Pulmonary Disease, NOS: non-obstructed smokers, NS: never-smokers.

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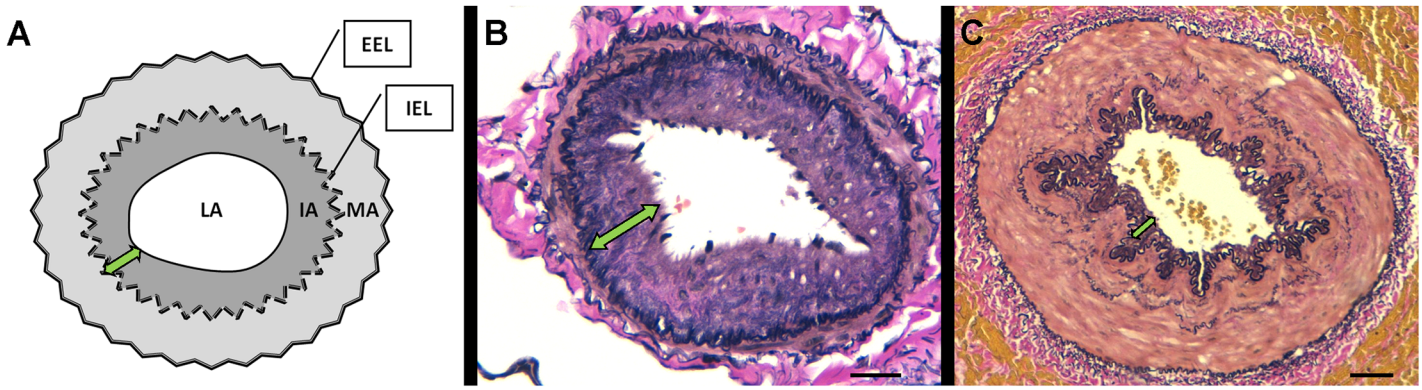

Fig 4. Morphometric and histologic studies. (A) Illustration of the methods used in morphometric analyses. The area enclosed by the continuous black line is the lumen area (LA), the area enclosed by internal elastic lamina (IEL), except for the LA, is the intima area (IA), and the area enclosed by the external elastic lamina (EEL) and IEL is the media area (MA). (B) Elastin-orcein stain of representative muscular pulmonary artery, scale bar = 20μm.(C) Elastin-orcein stain of representative intercostal artery, scale bar = 100 μm. The double-headed green arrows shows intimal thickening (IT) measured by the percentage of intimal area (%IA = 100X intimal area/ measured total area or area encompassed by the EEL). Note the difference between the IT of pulmonary (more remodeled arteries) and intercostal arteries.

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Circulating levels of PARC and inflammatory parameters

The circulating level of PARC was also measured by ELISA in the serum of all subjects. PARC concentrations were numerically higher in the COPD group but no statistically significant

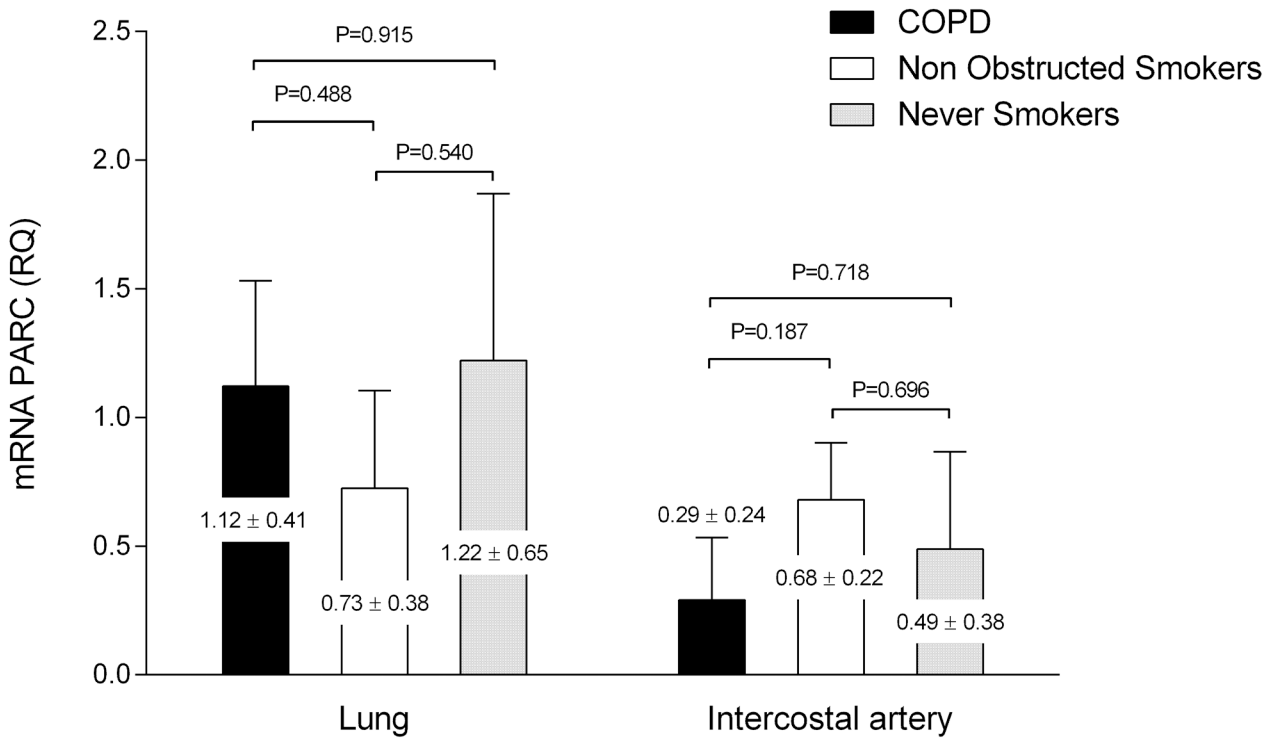

Fig 5. PARC gene expression in according to groups. Data of the PARC mRNA expression (fold change) in lung tissue and intercostal arteries according to groups. No significant differences were found between groups. Data are presented as LSM ± SEM. The reported p-value comes from the pair wise comparison with a general linear model using as covariables: gender, pack-years and the presence of diabetes mellitus.

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difference was observed in the comparisons between groups (100.4±19.7 for COPD, 96.6±19.8 for NOS, and 60.8±37.7 pg/ml for NS respectively, *P* overall = 0.718). In the present study, serum levels of C-reactive protein (CRP) and fibrinogen (Table 1) was determined as a measure of systemic inflammation. In order to explore the relationship between systemic inflammation and PARC expression, correlations analyses were performed between these inflammatory parameters and gene, protein and serum expressions of PARC. Of note, a weak correlation between CRP and PARC immuno-staining in alveolar parenchyma and fibrinogen and PARC gene expression at intercostal tissue was found (Spearman's Rho = 0.36, p = 0,036 and Spearman's Rho = 0.43, p = 0.022, respectively).

Discussion

The possible role of PARC in the pathophysiology of COPD has not been fully elucidated. In that sense, the results of the present study contribute to existing knowledge of the protein and gene expression of PARC in lung tissue and in the systemic vascular compartment in patients with COPD. The relevance of inflammation in the context of this chronic disease is beyond doubt [2,11]. This lung inflammation is characterized by both innate immunity (alveolar macrophages, neutrophils, dendritic cells, mast cells, eosinophils, natural killer cells) and adaptive immunity (T- and B-lymphocytes) [14]. However, alveolar macrophages appear to play a key role in orchestrating the inflammatory response [14] by secreting chemokines to attract immune cells, and in the case of PARC, T- lymphocytesin particular, from circulation and to the lung [8,9,15]. Based on that knowledge, we hypothesized that PARC is involved in the pro-inflammatory mechanisms of COPD. We report for the first time an increased protein expression of PARC in the lung tissue of COPD patients compared with non COPD subjects, suggesting a relationship between this chemokine and the development of the disease. These results are consistent with those of other studies of chronic lung diseases such as pulmonary fibrosis in relation to inflammation and disease activity [15–19]. However, the results of the present study do not suggest a higher mRNA PARC expression in both tissues (lung and intercostals arteries) in COPD subjects compared to non-COPD subjects. Also, we did not find any correlationship between gene and protein PARC, suggesting the existence of other possible mechanisms that dissociate the expression of mRNA into protein: spanning the transcription, processing and degradation of mRNAs to the translation, localization, modification and programmed destruction of the proteins themselves [20–21]. Nevertheless, the protein abundances observed reflect a dynamic balance among these processes [21].

Of note, we report through immunohistochemical analyses that this increased lung protein expression may occur especially in the SMCs of pulmonary muscular arteries and that this high expression is correlated with the severity of remodeling (intimal thickening). This suggests that the SMCs in the medial layer could be another source of PARC and that chemokine could be a mediator in these vascular changes. In studies of primary pulmonary hypertension, a profound pulmonary artery remodeling has been described that includes significant fibroproliferative and inflammatory changes to the entire vascular wall [22]. These findings support the idea that pulmonary hypertension results from a multistep process driven by the reprogramming of the gene-expression patterns that govern changes in cell metabolism, inflammation, and proliferation. Along this line, SMCs phenotypic changes have been described, specifically in the lung vascular remodeling of COPD [23] and in other primary pulmonary vascular diseases [22,24].

Previous studies described an increased expression of PARC in the human atherosclerotic plaques associated with the extent of changes and colocalizing with CD68-macrophages [25–26], with an approximate 100-fold increase in Types II and V lesions compared to normal

aortal tissue [25–26]. Therefore, because the COPD population has a clear risk of cardiovascular events and a higher prevalence of subclinical atherosclerosis [27–29], it was thought that one relevant aspect would be the evaluation of PARC expression in the intercostal arteries as a representation of systemic circulation in COPD subjects. However, our results fail to demonstrate differences between groups in PARC expression (both gene and protein) in IC arteries. These findings could be explained by the low inflammation and poor remodeling observed in the IC arteries compared to the pulmonary muscular arteries, where remodeling is more severe. Moreover, in our study the pattern expression of PARC in the IC arteries was predominantly observed in SMCs at the medial layer. These results are in agreement with other studies reporting the presence of chemokines such as MCP-1, MCP-4, and RANTES expressed in the SMCs of atherosclerotic vessels [30–33]. Although the expression of PARC does not appear to be enhanced in the initial vascular remodeling changes of IC arteries in patients with COPD, its possible role in the pathology of advanced vascular disease cannot be discarded, and has been described previously in the context of atherosclerosis [26].

Regarding the use of serum levels of PARC as a biomarker of activity in COPD, there are two large cohorts of COPD subjects (Lung Health Study and ECLIPSE with 4,825 and 1,809 subjects, respectively), which found that PARC was associated with mortality [11–12]. Furthermore, in the context of abdominal aortic aneurysms, PARC (both circulating levels in peripheral blood and gene expression) was associated with aortic lesions with a potential rupture risk [34]. In that sense, PARC could be useful as a serum marker of cardiovascular events in patients with vascular disease [34–35]. These data are in line with the results obtained in the present study, in which circulating levels of PARC have a numerical tendency to be higher in COPD subjects compared to other groups, though this trend did not reach statistical significance due to the high biological variability between subjects and the limited number of cases available for analysis.

Several limitations of this study need to be discussed. Firstly, the poor representation of female gender in the COPD and NOS groups due to the baseline characteristics of our population (patients with lung carcinoma and a major smoking habit are mostly male patients). This gender misbalance makes it difficult to draw conclusions about gender beyond spurious associations. Secondly, the population of the study has primary, treatable lung cancer; therefore lung cancer could be a possible introduced bias. However, we are assuming that any bias introduced because of lung carcinoma would be the same across all the subjects, since all the subjects included in the study suffer from lung carcinoma. Taking into account this limitation, we are able to compare PARC expression in order to find differences between COPD subjects and non-obstructed smokers, assuming that PARC expression in each group could be similarly influenced by the presence of lung cancer. It is important to consider that it would be impossible to obtain the demographic data, pulmonary function test and all the tissue specimens required in this study from subjects if they were not indicated for surgery. Tercially, the negative results found in circulating levels of PARC expression should be taken with caution, since a possible underpowered analysis due to small sample size and important biological variability could limit our results. Finally, due to the study's observational design, causal or strong conclusions cannot be drawn beyond observing an association between the presence of COPD and the expression of PARC.

Conclusions

In conclusion, the results of the present study suggest that PARC could have a relevant role in the development of vascular abnormalities in COPD, specifically in the lung, where the remodeled pulmonary vessels are present. However, other studies, especially experimental approaches, are needed to confirm these findings.

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Estudio III. Gene and Protein Expression of Fibronectin and Tenascin-C in Lung Samples from COPD Patients

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Gene and Protein Expression of Fibronectin and Tenascin-C in Lung Samples from COPD Patients

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Abstract
Purpose Fibronectin (Fn) and tenascin-C (TnC) are two extracellular matrix proteins associated with remodeling changes. Fn and TnC gene and protein expression in lung tissue, including their predominant location in bronchial and pulmonary artery structures, have not yet been fully evaluated. The aim of the present study was to assess: (1) gene expression of Fn and TnC in lung samples from chronic obstructive pulmonary disease (COPD) and non-COPD subjects; and (2) protein content and location of Fn and TnC in both groups.
Methods Consecutive subjects requiring lung resection due to lung cancer surgery were included. Lung specimens were examined for gene expression by quantitative real-time PCR (values expressed as fold change ratio). The analysis of their protein content and location was performed by western blot and immunohistochemical studies, respectively. Patients were divided into two cohorts according to COPD status.
Results A total of 41 patients (20 with COPD and 21 without COPD) were included. An enhanced Fn gene expression was observed in the COPD group compared to the non-COPD group (4.73 ± 0.54 vs. 2.65 ± 0.57; $P = 0.012$), whereas no differences in gene TnC expression were observed (2.91 ± 0.44 vs. 2.60 ± 0.48; $P = 0.633$). No differences in lung protein content and location were found between groups. Immunohistochemical evaluation showed a predominantly vascular and bronchial location of Fn and TnC in both groups.
Conclusions An enhanced lung gene expression of Fn was observed in COPD subjects compared to non-COPD subjects. No differences were found in Fn protein expression or in TnC gene or protein expression among groups.

Keywords Gene expression · Remodeling · Vascular · Extracellular matrix proteins

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Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive pulmonary changes with extrapulmonary consequences, as well as by a state of persistent local inflammation in most patients [1–4]. This ongoing chronic inflammation can trigger remodeling processes of pulmonary structures mediated by cytokines and growth factors [5–7]. The presence of vascular remodeling has been described from the early stages of the disease, even in patients with preserved lung function [8]. Of note, pulmonary vascular remodeling plays an important role in the subsequent development of pulmonary hypertension in COPD [9].

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Some of the extracellular matrix (ECM) proteins, such as fibronectin (Fn) and tenascin-C (TnC), have an active role in the inflammatory processes, contributing to re-building and repairing human tissues after injury [10–12]. However, there is little information available about the Fn and TnC gene re-expression under pathological conditions such as COPD. An increased gene expression of Fn at early stages of COPD and a progressive down-regulation in advanced stages of the disease has been described in lung samples [13]. Other immunohistochemical studies have observed an association between an increased TnC and Fn protein expression with the bronchial remodeling changes observed in asthma and COPD patients [14–16]. Noteworthy, these previous experiences were focused on describing only bronchial structures and lung parenchyma, whereas the vascular component of the lung (pulmonary arteries) was not analyzed in these studies [14–16].

To date, to the best of our knowledge, no study has evaluated simultaneously the pre- (gene) and post-transcriptional (protein) lung expression of Fn and TnC in samples from COPD patients, comparing the results with those from a cohort of patients without COPD. The objective of the present investigation was twofold: (1) to evaluate gene expression of Fn and TnC in lung samples from COPD and non-COPD subjects; and (2) to assess protein content and location of Fn and TnC in both groups.

Materials and Methods

Study Subjects

This was a prospective investigation conducted in consecutive subjects who required lung resection for treatment of primary lung cancer. All patients had history of current or former smoking habit of more than 10 pack-years, and were classified as (1) COPD patients and (2) non-COPD patients (criteria from the GOLD guidelines [1]). Exclusion criteria were the presence of any other pulmonary disease different to COPD and prior chemotherapy or radiotherapy treatment. All subjects underwent a post-bronchodilator spirometry and a test for determining diffusion lung capacity for carbon monoxide (DL_{CO}) prior to surgery. The study protocol was approved by the local ethics committee and performed in accordance with the Declaration of Helsinki. All patients provided written informed consent.

Sample Collection

Lung specimens were obtained from pieces of lung resection, as far away as possible from the tumor, and were processed immediately. A microscopic evaluation to confirm the absence of neoplastic cells was performed on each tissue sample before being included in the analysis.

Quantitative Real-Time-PCR

Quantitative real-time-PCR (qRT-PCR) was performed to determine the lung gene expression of Fn and TnC. In particular, the Fn isoform evaluated contains the type III extra domain A (ED-A), which is a specific marker involved in blood vessel morphogenesis [12], a crucial mechanism in remodeling processes [17]. In brief, tissue sections were stored in RNA stabilization reagent (RNA*later*, Qiagen) at −80 °C and RNA isolation was performed using the Trizol method. TaqMan Universal PCR Master Mix (Applied Biosystems) was used to amplify the cDNA. Using the $2^{\Delta\Delta C_t}$ method, the mRNA expression of the target genes was normalized for the expression of housekeeping genes (18S rRNA and DNA-directed RNA polymerase II). A TaqMan assay was designed for ED-A variant of Fn, using the following sequence: forward primer, AGGACTGGCATTCA CTGATGTG; reverse primer, GTGGGCTTTCCCAAGC AA; and probe, ATGTCGATTCCATCAAA. Commercial inventoried TaqMan assays were used for TnC (Applied Biosystems TaqMan Assay, Hs01115665_m1) and 18S (Applied Biosystems Taqman Assay, Hs99999901_s1). A common calibrator for each plate was used. Data are reported as a relative quantification (fold change ratio) of mRNA of Fn and TnC from each sample.

Immunohistochemistry

The Fn and TnC location in lung tissue was examined by immunohistochemistry methods. Serial sections of paraffin-embedded lung tissue were immunostained overnight with monoclonal antibodies against Fn (ED-A, 1/400, AB6328; Abcam) and TnC (1/300, AB3970; Abcam) using the avidin–biotin complex/peroxidase (Vectastain Elite ABC kit, Vector Laboratories). Immunoreaction analysis was evaluated in: (1) the pulmonary muscular arteries (100–500 μm): endothelial cells and smooth muscles cells (SMCs); (2) the bronchial structures: epithelial cells layer, sub-epithelial baseline membrane (SEBM), and airway smooth muscle cells (ASMCs); and (3) lung parenchyma. The pulmonary arteries and bronchial structures with positive immunoreaction were expressed as a percentage of the total arteries and bronchus examined, respectively. Lung parenchyma with positive immunoreaction was expressed as the percentage of stained area. The intensity of the positive immunoreaction was graded as 1, 2, and 3 meaning mild, moderate, and intense staining, respectively. An average grading score was computed for arteries and bronchial structures in each subject.

Western Blot Assays

The protein content of lung tissue was examined by western blot analysis. Previously, the Lowry method was used to quantify the protein extracts. In short, an amount of 100 μg of protein was denatured and placed in a sodium dodecyl sulphate polyacrylamide gel (7 %) for electrophoresis to be subsequently transferred to nitrocellulose membrane (Bio-Rad and Mini protean II). Overnight incubation at 4 °C was performed with monoclonal primary antibodies against ED-A Fn (1/300 dilution, AB6328, Abcam) and TnC (1/150 dilution, AB3970; Abcam). After 1-h incubation with secondary antibody (Ab. anti-IgG1; R&D Systems), specific immunoreactivity was detected with the avidin–biotin–peroxidase complex method (Vector). Protein bands were visualized by enhanced chemiluminescence (Supersignal Western, Pierce) and digitized using the Image Reader LAS-3000 (Fuji). Multi-gauge v1.3 Software was used to quantify the densitometry of the protein band intensity. The ratio of the band intensity (target protein/β-actin, A1978; Sigma) was used as a measure of the protein content in each sample.

Study Endpoints and Sample Size Calculation

The primary endpoint of this study was the fold change ratio of lung gene expression (ED-A Fn or TnC) in COPD patients compared to non-COPD patients. Assuming a standard deviation of 2.0, a sample size of 18 subjects per group was needed to detect a difference between groups of the usual arbitrary fold change cutoff of 2 (minimal significant difference for gene expression analysis) [18]; with 85 % power and a two-tailed α-value less than 0.05. Considering an approximate 17 % dropout rate (e.g., insufficient RNA obtained for PCR), inclusion of 44 subjects was allowed to ensure that gene expression data from 18 patients were available for analysis. Secondary endpoints included between-group comparisons for: (1) percentage and intensity of positive immunoreaction for Fn and TnC in arterial and bronchial structures, and (2) protein content of Fn and TnC in lung tissue.

Statistical Analysis

For baseline characteristics, continuous variables were expressed as mean ± SD or median and interquartile range whether a normal distribution was assumed or not (Kolmogorov–Smirnov test), respectively. Comparisons of continuous variables were performed with Student's *t* test or Mann–Whitney's *U* test as appropriate, while qualitative variables were compared with Chi square test or Fisher's exact test (any expected value <5). An ANOVA method with a general linear model was used to evaluate the primary endpoint and all other between-group comparisons. Adjusted analyses were performed with an ANCOVA method, using as covariates unbalanced demographic or clinical variables ($P < 0.10$) or those variables considered relevant. Finally, covariates included were age, gender, current smoking habit, and use of inhaled corticosteroids and statins. Since active smoking and the use of inhaled corticosteroids have been associated with regulation of Fn and/or TnC expression [19–23], exploratory stratified analyses were performed in order to assess the possible effect of these variables on gene expression. A two-tailed *P* value of <0.05 was considered to indicate a statistically significant difference. Results are reported as least squares mean (LSM) ± standard error of the mean (SEM) for the above detailed analyses. Statistical analysis was performed using the PASW Statistics v18.0 software (SPSS Inc., Chicago, IL).

Results

Consecutive samples from 44 patients undergoing lung resection surgery were included in the study, of which three of them were discarded due to poor quality of the sample or insufficient RNA obtained. Therefore, 41 patients were included in the present analysis, 20 COPD subjects (12 former smokers and 8 current smokers), and 21 non-COPD patients (12 former smokers and 9 current smokers). There were no significant differences in baseline characteristics (Table 1) between groups, except for gender and age.

Gene Expression

An enhanced Fn gene expression was observed in the COPD group compared to the non-COPD group (4.73 ± 0.54 vs. 2.65 ± 0.57; unadjusted *P* value = 0.012 and adjusted *P* value = 0.030). Conversely, no difference was observed for TnC gene expression (2.91 ± 0.44 vs. 2.60 ± 0.48; unadjusted *P* value = 0.633 and adjusted *P* value = 0.669). In addition to COPD status, a current smoking habit was the only covariate significantly associated with Fn gene expression in the adjusted analysis ($P = 0.019$), observing an inverse relationship of current smoking habit with Fn expression. In the stratified analysis considering both COPD and smoking status, a higher Fn gene expression was found in the subset of patients with COPD who were former smokers (Fig. 1a), which was statistically significant when compared to COPD current smokers ($P = 0.032$). No differences were observed in TnC gene expression when evaluating smoking habit (Fig. 1b) as well as no differences in Fn or TnC gene expression were observed regarding the use of ICS in COPD patients (data not shown).

Table 1 Baseline characteristics and lung function parameters of the study groups

Characteristic	Non-COPD ($n = 21$)	COPD ($n = 20$)	P value
Male gender, *n* (%)	16 (76.2)	20 (100)	0.048
Age (years)[a]	61.1 ± 9.13	67.5 ± 6.10	0.011
BMI (kg/m^2)	25.1 [23.4–27.4]	27.9 [24.2–29.6]	0.060
Pack-years[a]	37.4 ± 20.3	46.1 ± 20.3	0.182
Current smoking, *n* (%)	9 (42.9)	8 (40.0)	0.853
Years stopped smoking[a]	11.6 ± 4.1	14.3 ± 2.3	0.323
COPD stage I/II, *n* (%)	–	7 (35)/13(65)	–
Pulmonary hypertension, *n* (%)	0 (0)	2 (10.0)	0.487
Diabetes Mellitus, *n* (%)	3 (14.3)	3 (15.0)	1.000
Dyslipidemia, *n* (%)	7 (33.3)	10 (50.0)	0.279
Systemic hypertension, *n* (%)	10 (47.6)	12 (60.0)	0.427
Coronary arterial disease, *n* (%)	1 (4.8)	2 (10.0)	0.606
Statins, *n* (%)	7 (33.3)	9 (45.0)	0.444
ICS, *n* (%)	0(0)	8 (40.0)	0.001
FVC, % predicted	94.0 [80.1–106.7]	95.1[81.4–114.1]	0.896
FEV_1, % predicted	91.0 [84.1–99.9]	73.2 [62.9–91.1]	0.003
FEV_1/FVC, %	76.0 [72.7–79.2]	63.1 [57.7–67.6]	<0.001
DL_{CO}, % predicted	82.6 [73.6–88.0]	75.9 [59.8–93.9]	0.360

Data reported as mean ± SD or median [IQR]

BMI body mass index, *ICS* inhaled corticosteroids, *FVC* forced vital capacity, FEV_1 forced expiratory volume in one second, DL_{CO} diffusion lung capacity for carbon monoxide

P value of <0.05 was considered statistically significant

[a] Variables with a normal distribution

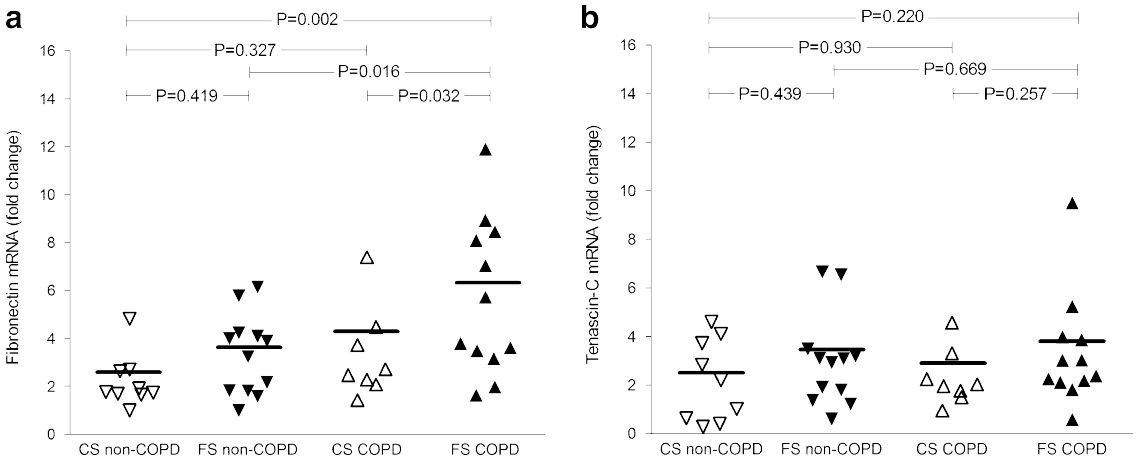

Fig. 1 Lung gene expression according to smoking habit and COPD and non-COPD groups. Individual data of the mRNA expression (fold change) of fibronectin (**a**) and tenascin-C (**b**) in lung tissue, according to groups and smoking habit. *Horizontal bars* indicate least squares mean values. *CS* current smokers, *FS* former smokers

Protein Location

Immunoreaction results are summarized in Table 2. In pulmonary arteries, Fn was predominantly expressed in endothelial cells and SMCs, whereas TnC mainly in SMCs. In bronchial structures, positivity for Fn and TnC was predominantly observed in the cytoplasm of epithelial cells; with mild or no expression in SEBM or ASMCs. A positive immunoreaction for Fn and TnC was also observed on alveolar epithelium and capillaries of the alveolar wall, constituting more than 60 % of stained area of the lung parenchyma (66.4 ± 7.1 % and 70.1 ± 12.2 % for Fn and TnC, respectively). No differences in the percentage of positivity or the intensity score between COPD and non-

Table 2 Immunohistochemical lung expression of ED-A fibronectin and tenascin-C by groups

Characteristic	ED-A fibronectin			Tenascin-C		
	Non-COPD	COPD	*P* value	Non-COPD	COPD	*P* value
N° arteries by patient[a]	16.4 ± 5.6	15.9 ± 6.8	0.821	14.0 ± 6.3	14.6 ± 7.2	0.843
Positive immunoreaction in ECs (%)	100 [97.1–100]	100 [97.2–100]	0.976	0 [0–27.6]	0 [0–14.1]	0.796
Positive immunoreaction in SMCs (%)	66.7 [27.9–96.3]	78.6 [59–87.5]	0.376	42.6 [16.8–67.5]	34.3 [18.1–64.4]	0.790
Intensity of the immunoreaction in ECs	1.2 [1–2]	1.4 [1–1.8]	0.738	1 [0.5–1]	1 [1–1.1]	0.429
Intensity of the immunoreaction in SMCs	1 [1–1.9]	1 [1–1.3]	0.556	1 [1–1.2]	1 [1–1.4]	0.931
N° bronchial structure by patient[a]	5.6 ± 3.2	5.9 ± 4.2	0.821	5.8 ± 2.4	5.4 ± 3.8	0.835
Positive immunoreaction in BE (%)	83.3 [43.7–100]	100 [92.5–100]	0.129	80 [33.3–100]	100 [75–100]	0.393
Positive immunoreaction in SEBM (%)	12.5 [0–16.6]	12.9 [0–18.6]	0.808	20 [0–100]	37.5 [0–70.8]	0.877
Positive immunoreaction in ASMc (%)	12.5 [0–27.1]	10 [0–37.1]	1.0	14.3[0–20]	10 [0–14.6]	0.311
Intensity of the immunoreaction in BE	1 [1–1]	1 [1–1.1]	0.508	1 [1–1]	1 [1–1]	0.831
Intensity of the immunoreaction SEBM	1 [1–1.1]	1 [1–1.3]	0.662	1 [1–1.5]	1 [0–1]	0.400
Intensity of the immunoreaction ASMc	1 [1–1.2]	1 [1–1.3]	1.0	1 [0–1]	1 [0–1]	0.244
Lung parenchyma						
Positive immunoreaction (%)	69.0 [59.1–72]	62.0 [59.0–72.1]	0.403	70.0 [60.5–79.5]	76.5 [69.3–79.0]	0.976
Intensity of the immunoreaction	2 [1–3]	3 [1.5–3]	0.284	1 [1–2]	1 [1–2]	0.670

Data reported as median [IQR] or mean ± SD

ED-A extra domain A of fibronectin, *ECs* endothelial cells, *SMCs* smooth muscle cells, *BE* bronchial epithelium, *SEBM* sub-epithelial baseline membrane, *ASMc* airway smooth muscle cells

A 2-tailed probability value of $P < 0.05$ was considered to be statistically significant

[a] Variables with a normal distribution.

The pulmonary arteries and bronchial structures with positive immunoreaction were expressed as a percentage of the total arteries and bronchus examined, respectively. Lung parenchyma with positive immunoreaction is expressed as the percentage of stained area. The intensity of the positive immunoreaction was additionally graded as 1, 2, and 3 meaning mild, moderate, and intense staining, respectively

COPD patients were observed at any location. Representative photomicrographs of immunohistochemical expression of Fn and TnC in lung tissue from COPD and non-COPD subjects are represented in Figs. 2 and 3, respectively.

Protein Content

Western blot analysis was performed to confirm the presence of Fn and TnC protein content in total lung tissue, previously assessed in the immunohistochemical analyses described above. No differences were observed for the protein signal of Fn (0.89 ± 0.14 vs. 0.80 ± 0.16; $P = 0.661$) and TnC (1.27 ± 0.19 vs. 1.20 ± 0.19; $P = 0.806$) between COPD and non-COPD subjects in the densitometric analysis (Fig. 4) of the protein bands.

Discussion

In the present investigation, we assessed both the gene and the protein expression of two relevant ECM components, Fn and TnC, in the lung tissue of a group of patients with mild or moderate COPD and a group of non-COPD subjects (smokers with normal lung function). Previous evidence for the gene re-expression of these two remodeling proteins in patients with COPD is scarce. In particular, Gosselink and colleagues observed a decreased expression of Fn, among other remodeling proteins, inversely related to FEV_1, which suggests a progressive down-regulation of Fn gene expression associated with lung function worsening and, thus, progression of the disease [13]. In the present study, an up-regulation of Fn gene expression was observed in total lung tissue extracted from COPD patients compared to non-COPD subjects. Of note, all patients included in the analysis had a mild or moderate form of the disease and a preserved DL_{CO}, which cannot rule out the presence of mild emphysema but denotes a lack of significant parenchyma destruction and that these patients had not developed severe emphysema. These findings might support the hypothesis of an initial enhanced gene expression of some ECM proteins such as Fn at early stages of COPD, being involved in initial vascular and airway remodeling changes, which decreases with severity of emphysema and progression of the disease, and is overall in line with results from prior investigations [13, 24]. On the contrary, a significant increase in the gene

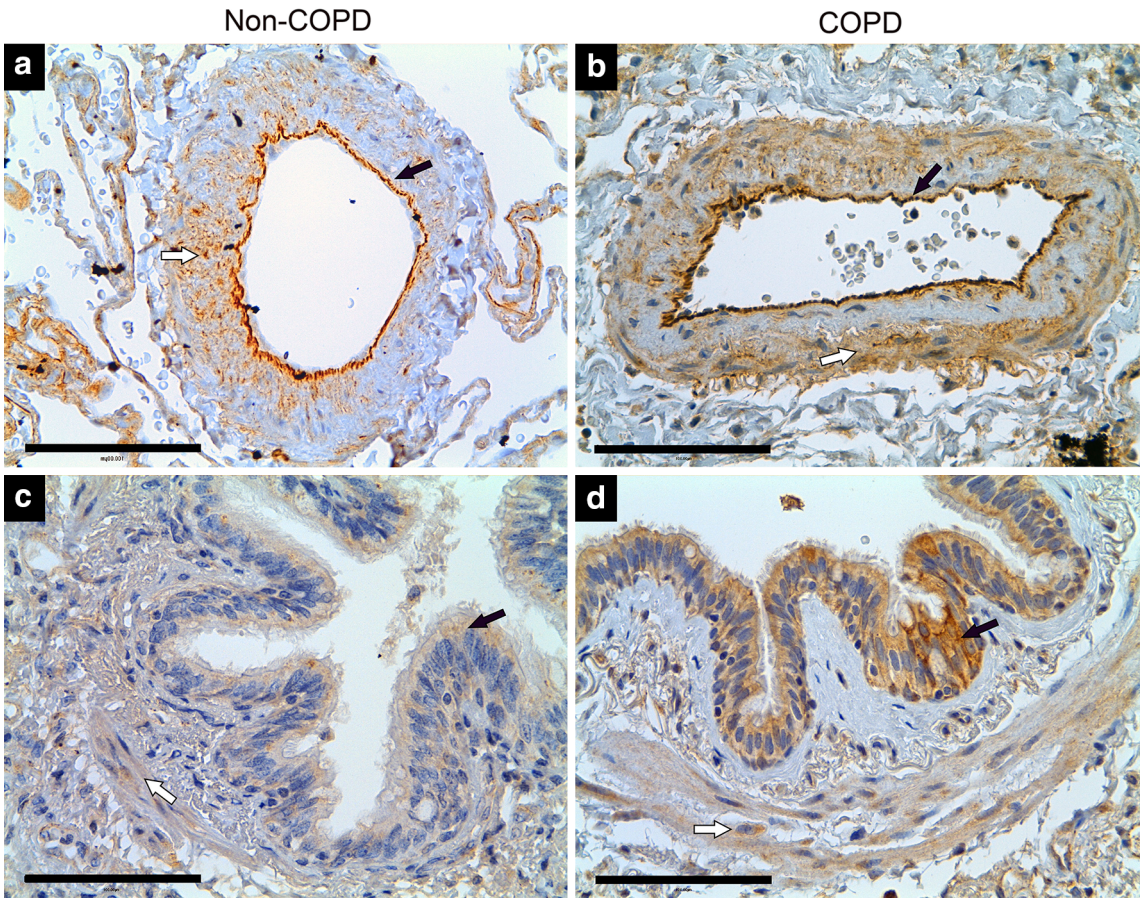

Fig. 2 Representative photomicrographs of immunohistochemical expression of ED-A fibronectin in lung tissue from non-COPD and COPD subjects. Image **a** represents a pulmonary muscular artery from a non-COPD subject, which has a positive immunoreaction in endothelial cells layer (*black arrow*) and a mild expression in smooth muscles cells from medial layer (*white arrow*). Image **b** shows a pulmonary artery from a COPD subject with a positive immunoreaction in endothelium (*black arrow*); and also in the medial layer (*white arrow*). Images **c** and **d** represent bronchial structures from a non-COPD and COPD subject, respectively, with a positive immunoreaction in the cytoplasm from epithelial cells (*black arrows*), and a mild immunoreaction in airways smooth muscles cells (*white arrows*). *Scale bar* 100 μm

expression of TnC, which has been associated with remodeling processes in several settings [11, 12, 25], was not seen in COPD patients compared to non-COPD subjects in the present investigation. This could be attributed to a differential gene expression of TnC that may depend on the underlying disease. In fact, a recent investigation by Hoffmann and colleagues observed a distinct gene expression pattern of TnC in patients with pulmonary hypertension, comparing subjects with COPD to individuals with idiopathic pulmonary fibrosis, which suggests that pulmonary arterial remodeling is caused by different molecular mechanisms that may vary depending on the specific pulmonary disease [26]. Whether these findings might overall suggest a predominant role of Fn in the initial changes leading to remodeling of arteries in COPD patients warrants further investigation.

Other factors such as smoking habit and the use of corticosteroids have been associated with regulation of gene and protein expression of several ECM components under in vitro conditions (fibroblasts culture) or in tissue samples [19–23, 27]. In particular, it has been reported in a mechanistic study that the volatile components of cigarette smoke in acute exposures inhibit lung fibroblast proliferation and decrease the production of some ECM proteins such as Fn [19], which could be of relevance in terms of progression of COPD. This is in line with the findings of the present investigation, since we observed a significant decrease of Fn gene expression associated with current smoking habit in the overall population of the study, which was statistically significant in the subset of patients with COPD. To the best of our knowledge, the association of smoking habit with TnC gene expression in COPD or non-COPD subjects has not been evaluated previously, and we failed to find any difference between groups in the present investigation. Despite the fact that there are to date very few data available, it has been suggested that corticosteroids may modulate extracellular matrix composition in

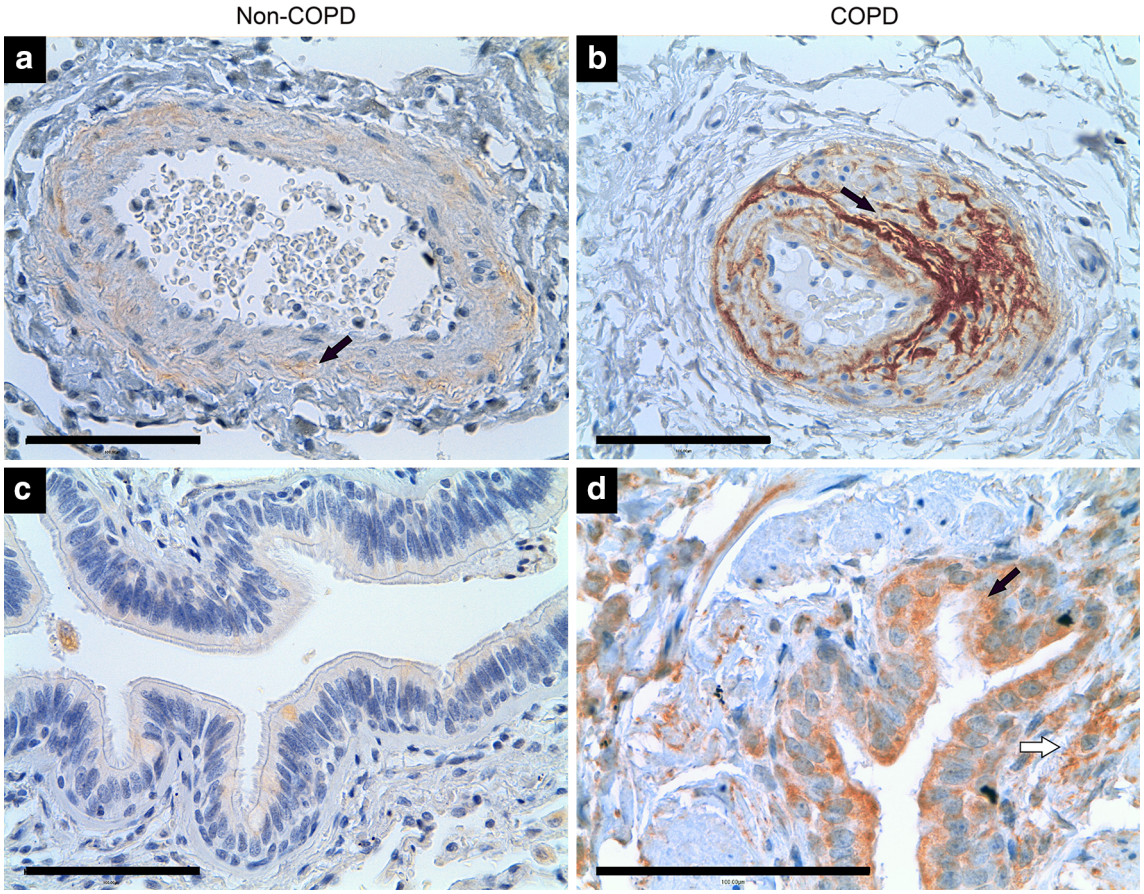

Fig. 3 Representative photomicrographs of immunohistochemical expression of tenascin-C in lung tissue from non-COPD and COPD subjects. Image **a** represents a pulmonary muscular artery from a non-COPD subject, which has a negative immunoreaction at endothelial cells and a mild expression in the medial layer (*black arrow*). Image **b** shows a remodeled pulmonary artery from a COPD subject with a negative immunoreaction at endothelial cells and an intense positive immunoreaction at medial layer (*black arrow*). Images **c** and **d** represent bronchial structures from a non-COPD and COPD subject, respectively, with a negative immunoreaction (**c**) at epithelial cells and airways smooth muscles cells in non-COPD subject. A positive immunoreaction (**d**) is represented in the cytoplasm from epithelial cells (*black arrow*) and airways smooth muscles cells (*white arrow*). *Scale bar* 100 μm

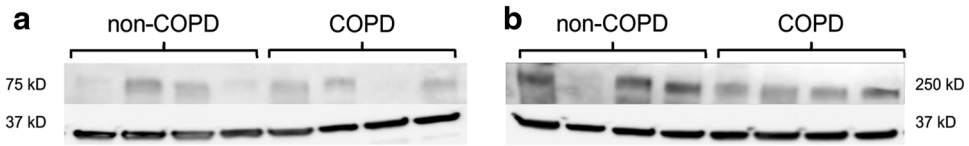

Fig. 4 Protein content of fibronectin and tenascin-C in lung tissue according to groups. **a** Western blot analysis for fibronectin in lung tissue of non-COPD subjects and COPD subjects. Bands at 75 and 37 kDa are consistent with size of fibronectin and β-actin, respectively. **b** Western blot analysis for tenascin-C in lung tissue of non-COPD subjects and COPD subjects. Bands at 250 and 37 kDa are consistent with the size of tenascin-C and β-actin, respectively

lung diseases [21–23]. A single in vitro investigation has observed that a corticosteroid (fluticasone) may decrease TnC and ED-A Fn expression in cultures of human fibroblasts [21]. Using a different approach, we did not observe a different pattern of gene expression in COPD subjects undergoing inhaled corticosteroid treatment in the present investigation.

A novel finding of the present study is the evidence of protein expression of Fn and TnC in the pulmonary arteries from COPD subjects and non-COPD smokers, as these proteins are not usually detected in normal tissue [12, 28]. TnC expression at the intima and media layer of remodeled pulmonary vessels from idiopathic pulmonary fibrosis subjects with pulmonary hypertension has been recently reported [26]. Interestingly, an up-regulation of TnC gene expression was observed in these patients when compared to COPD subjects [26]. In addition, a higher protein expression of Fn and TnC has been associated to a gradient

for progressive vascular remodeling processes in patients with pulmonary hypertension due to congenital heart diseases [29]. Overall, these data support the idea that these ECM proteins could play a role in early vascular remodeling processes, even in smokers with normal lung function, although the molecular mechanisms may vary according to underlying pathologies.

We acknowledge the inherent limitations of this investigation due to its observational design and a small sample size, as the study was powered to detect differences in the primary end point but not for multiple comparisons. In addition, the lack of a control group (never smokers) makes it difficult to draw definitive conclusions about the influence of chronic tobacco exposure in this setting. However, we included subjects with past and current smoking habit, which provide interesting inputs regarding the influence of acute smoke exposure in gene expression.

In summary, an enhanced lung gene expression of Fn was observed in COPD subjects compared to non-COPD subjects, with differences in the Fn expression under smoking conditions. Furthermore, there is a lung vascular protein expression of Fn and TnC, even in smokers.

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Conflict of interest The authors declare that they have no others conflicts of interest.

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Estudio IV. Impact of acute exacerbations on platelet reactivity in COPD patients with and without antiplatelet therapy

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ORIGINAL RESEARCH

Impact of acute exacerbations on platelet reactivity in chronic obstructive pulmonary disease patients

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Background: A higher risk of atherothrombotic cardiovascular events, which are platelet-driven processes, has been described during acute exacerbations of chronic obstructive pulmonary disease (AECOPD). However, the relevance of platelet reactivity during AECOPD and whether this is affected by antiplatelet agents are not fully elucidated to date. This study aimed to evaluate whether platelet reactivity is augmented during an exacerbation in COPD patients with and without antiplatelet therapy and its association with systemic inflammatory parameters.

Materials and methods: Prospective, observational, ex vivo investigation was conducted in consecutive patients suffering an exacerbation of COPD. Platelet reactivity was assessed during AECOPD and at stable state. Platelet function assays included: 1) vasodilator-stimulated phosphoprotein assay expressed as $P2Y_{12}$ reactivity index (PRI), 2) multiple electrode aggregometry and 3) optical aggregometry. Systemic inflammatory parameters such as leukocyte count, interleukin-6 and fibrinogen were also assessed.

Results: Higher platelet reactivity was observed during AECOPD compared to stability measured by vasodilator-stimulated phosphoprotein (PRI: 75.2%±1.9% vs 68.8%±2.4%, p=0.001). This augmented platelet aggregability was also observed in the subset of patients on antiplatelet therapy (PRI: 72.8%±3.1% vs 61.7%±7.5%, p=0.071). Consistent findings were observed with all other platelet function tests. Patients with greater enhancement of inflammatory markers during AECOPD were more likely to present a higher increase in platelet reactivity.

Conclusion: Platelet reactivity is increased during AECOPD, which may contribute to the augmented cardiovascular risk of these patients. Additionally, the increase in platelet reactivity might be associated with an increment in inflammatory markers during exacerbations.

Keywords: airflow limitation, inflammation, platelet aggregation, antiplatelets agents, thrombosis

Introduction

Cardiovascular disease is the most important comorbidity associated with chronic obstructive pulmonary disease (COPD) due to its impact on overall prognosis including mortality.[1–3] Systemic inflammation has been suggested as one of the main factors that play a significant role in the pathogenesis of atherothrombosis in COPD,[4,5] which is characterized by the presence of low-grade systemic inflammation in the stable state[6] and, noteworthy, by an acute inflammatory response during exacerbations of the disease, as shown by Thomsen et al.[7] The increased systemic inflammation associated with acute exacerbations is of particular relevance since it can be an underlying mechanism that may contribute to the impaired cardiovascular outcomes observed in COPD patients. In line with this, atherothrombotic complications such as myocardial

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infarction or stroke are particularly augmented during an acute exacerbation of COPD (AECOPD).[8,9]

Platelets are key elements in the development of atherosclerosis and its atherothrombotic complications.[10] Several studies have observed that systemic inflammation may contribute to platelet activation and, thus, to an increased cardiovascular risk.[11,12] In addition, an interesting mechanistic investigation by Maclay et al has shown increased platelet activation in COPD patients compared to non-COPD subjects, and during AECOPD compared to stability.[13] However, the magnitude of the increment in platelet reactivity during an AECOPD is not fully elucidated to date, and if this is affected by antiplatelet agents has not been previously evaluated. The aim of this study was to evaluate whether platelet reactivity is augmented during an AECOPD compared with stability in COPD patients with and without antiplatelet therapy (APT) and its possible association with systemic inflammatory parameters.

Materials and methods

Population and study design

This was a prospective, ex vivo, observational investigation conducted in consecutive COPD patients attended during an exacerbation at the pulmonology day hospital from a tertiary centre. All patients were between 40 and 80 years of age and had been diagnosed of COPD following Global initiative for chronic Obstructive Lung Disease recommendations.[1] An AECOPD was defined as an acute event characterized by worsening of the patient's respiratory symptoms that is beyond normal day-to-day variations, which leads to a change in medication.[1] Exacerbations were classified as moderate (outpatient treatment requiring antibiotics and/or oral corticosteroids) and severe (requiring hospital admission).[14] Exclusion criteria were the presence of other pulmonary disease, current therapy with anticoagulant agents or glycoprotein IIb/IIIa inhibitors, any active malignancy or hematologic disease, platelet count $<100\times10^6/\mu L$, severe chronic kidney disease (creatinine clearance <30 mL/min) and pregnant females. Patients could receive APT with aspirin or clopidogrel for concomitant chronic cardiovascular conditions.

The study had a prospective design with paired data, in which assessments were performed at two time points: the first one during AECOPD and the second one after the patient had recovered stability (4–6 weeks after the first visit and no symptoms of exacerbation or administration of antibiotics or oral corticosteroids for at least 2 weeks). In patients receiving APT, blood samples were collected for platelet function testing before the daily maintenance dose of aspirin or clopidogrel was administered. The study was approved by the local ethics committee "Comitè Ètic d' Investigació Clínica Del Hospital de Bellvitge", No PR077/13, and performed in accordance with the Declaration of Helsinki. All subjects provided written informed consent to the study.

Platelet function assays

Blood samples were collected from antecubital vein, discarding the first 2–4 mL of blood to avoid spontaneous platelet activation. Samples were processed by trained laboratory personnel blinded to the patient status and medication within 2 hours of blood drawing. Several agonists were used in platelet function assessments in order to explore different platelet activation signaling pathways. In patients receiving APT, aspirin-mediated effects were specifically evaluated by using arachidonic acid and collagen as agonists, while clopidogrel-mediated effects were specifically assessed by using adenosine diphosphate (ADP) as an agonist. Platelet function assays included flow cytometric analysis of the phosphorylation status of vasodilator-stimulated phosphoprotein (VASP), light transmission aggregometry (LTA) and multiple electrode aggregometry.

Analysis of the phosphorylation status of VASP

The $P2Y_{12}$ reactivity index (PRI) measures the functional status of the platelet $P2Y_{12}$ signaling pathway and was determined according to standard protocols.[15] In brief, VASP phosphorylation (VASP-P) was measured by quantitative flow cytometry using commercially available labeled monoclonal antibodies (Biocytex Inc., Marseille, France). The PRI was calculated after measuring the mean fluorescence intensity (MFI) of VASP-P levels following challenge with prostaglandin E1 (PGE1) and PGE1 + ADP. PGE1 increases the VASP-P levels through stimulation of adenylate cyclase, and ADP binding to purinergic receptors leads to inhibition of adenylate cyclase; thus, the addition of ADP to PGE1-stimulated platelets reduces the levels of PGE1-induced VASP-P. The PRI was calculated as follows: ([MFI PGE1]–[MFI PGE1 + ADP]/[MFI PGE1]) ×100%. An increased PRI is indicative of higher platelet reactivity.

LTA

LTA was performed according to standard protocols as previously described.[16] Briefly, blood-citrate tubes were centrifuged at 100× *g* for 10 min to recover platelet-rich plasma (PRP) and further centrifuged at 2,400× *g* for 15 min to recover platelet-poor plasma (PPP). Platelet aggregation was assessed using PRP and PPP by the turbidometric method in a two-channel aggregometer (Chrono-Log 490 Model;

Chrono-Log Corp., Havertown, PA, USA). Light transmission was adjusted to 0% for PRP and 100% for PPP for each measurement. Maximal platelet aggregation is reported as a percentage and was measured following stimuli with several agonists: ADP (5 and 20 μmol/L), arachidonic acid (1 mmol/L) and collagen (2 μg/mL).

Multiple electrode aggregometry

Blood was collected in hirudin-treated tubes. Multiple electrode aggregometry was performed in whole blood with the Multiplate™ analyzer (Hoffman-La Roche Ltd., Basel, Switzerland) as previously described.[17] This instrument assesses the change in impedance caused by the adhesion of platelets onto sensor units formed by silver-covered electrodes. In this investigation, the following agonists were used: ADP (6.4 μmol/L), arachidonic acid (0.5 mmol/L), collagen (3.2 μg/mL) and thrombin receptor activating peptide (32 μmol/L). Aggregation curves were recorded for 6 min and platelet aggregation was determined as the area under the curve of arbitrary aggregation units (AU × min).

Systemic inflammatory parameters

Three inflammatory parameters involved in COPD exacerbations were assessed: leukocyte count, interleukin-6 (IL-6) and fibrinogen.[7] Leukocytes were measured as part of complete cell blood counts performed at the time points described above. To quantify the IL-6 (expressed in pg/mL) and fibrinogen (expressed in g/L) serum levels, individual enzyme-linked immunosorbent assay tests were used following the manufacturer's instructions. The lower limit of detection for IL-6 was 0.156 pg/mL (HS600B; R&D Systems, Inc., Minneapolis, MN, USA) and for fibrinogen was 1.56 ng/mL (ab208036; Abcam, Cambridge, UK).

Study endpoints and sample size

The primary endpoint of this study was the difference in platelet reactivity measured as PRI of COPD patients during the AECOPD compared to stable state. Assuming an SD of 20%, a sample size of 36 subjects was needed to detect a minimal difference between time points of 10%, with 85% power and a two-tailed p-value <0.05 for a paired data comparison. Considering an approximate 10% dropout rate (eg, inadequate samples or loss in the follow-up), the inclusion of 40 subjects was allowed to ensure that data from 36 patients were available for analysis. The secondary endpoints included: 1) differences in platelet reactivity measured with the other platelet function assays during the AECOPD compared to stable state and 2) changes in inflammatory parameters during the AECOPD compared to stable state and their association with platelet reactivity.

Statistical analysis

Conformity to the normal distribution was evaluated for continuous variables with the Kolmogorov–Smirnov and the Shapiro–Wilk tests. For baseline characteristics, categorical variables were expressed as frequencies and percentages, whereas continuous variables were summarized by mean ± SD or by median (interquartile range) if a normal distribution could be assumed or not, respectively. A repeated-measures analysis of variance (ANOVA) model was used to evaluate the primary endpoint and to make all other intragroup comparisons between the AECOPD compared to the stable state. In order to evaluate the association between inflammation and platelet aggregability, exploratory analyses comparing the change in platelet reactivity measured by VASP (ΔPRI: PRI during exacerbation–PRI during stability) according to tertiles of the difference (Δ) of each inflammatory marker (levels during exacerbation–levels during stability) were performed using an ANOVA method with a general linear model. Results are reported as least squares mean ± standard error of the mean for the above-detailed ANOVA analyses. A p-value <0.05 was considered statistically significant for all comparisons. Statistical analysis was performed using SPSSv18.0 software (SPSS Inc., Chicago, IL, USA).

Results

Forty consecutive severe COPD patients consulting with an AECOPD were prospectively included. Two patients were excluded because they were lost in the follow-up, and one patient was excluded because anticoagulant therapy for atrial fibrillation was prescribed before visit 2 occurred. Therefore, a total of 37 patients were included in this analysis: 29 (78.4%) with a moderate AECOPD and 8 (21.6%) with a severe AECOPD, thus requiring admission. In this cohort, 10 patients receiving APT were included: 8 on aspirin and 2 on clopidogrel. Demographic and clinical characteristics of the overall population are summarized in Tables 1 and 2. Characteristics of the AECOPD as well as treatment approach are listed in Table 3.

Platelet reactivity during an AECOPD

Higher platelet reactivity was observed during AECOPD compared to stability, as measured by VASP (PRI: 75.2%± 1.9% vs 68.8%±2.4%, p=0.001) in the overall sample (Figure 1). Consistent results were obtained with the other platelet function assays performed and all agonists used, which showed a numerically greater platelet reactivity

Table 1 Demographic and clinical characteristics

	N=37
Age (years), mean ± SD	69.8±5.7
Male, n (%)	35 (94.6)
Body mass index (kg/m^2), mean ± SD	27.7±5.1
Risk factors and medical history	
Current smoking, n (%)	4 (10.8)
Pack-years, median (IQR)	45.0 (37.0–68.5)
Hypertension, n (%)	22 (59.5)
Diabetes mellitus, n (%)	4 (10.8)
Dyslipidemia, n (%)	16 (43.2)
Coronary heart disease, n (%)	5 (13.5)
Chronic heart failure, n (%)	6 (16.2)
OSAS, n (%)	7 (18.9)
Previous stroke, n (%)	2 (5.4)
Peripheral vascular disease, n (%)	2 (5.4)

Abbreviations: IQR, interquartile range; OSAS, obstructive sleep apnea syndrome.

during exacerbations, although statistical significance was not reached in all of them (Table 4).

The increase in platelet reactivity during exacerbations was consistent in patients with or without concomitant APT. In patients not receiving APT, a statistically significant greater PRI was observed during AECOPDs compared to stability (76.0%±1.5% vs 71.2%±2.0%, p=0.007; Figure 1), with parallel findings obtained with the other platelet function tests used (Table 4).

Table 2 Baseline pulmonary function parameters, laboratory data and medical therapy

	N=37
Pulmonary function test	
FEV_1 post-bronchodilator % predicted, median (IQR)	38.6 (30.0–45.5)
FEV_1/FVC post-bronchodilator, median (IQR)	38.5 (35.4–45.7)
Medical therapy	
Aspirin, n (%)	8 (21.6)
Clopidogrel, n (%)	2 (5.4)
Statins, n (%)	14 (37.8)
PPI, n (%)	24 (64.9)
Oral antidiabetic agents/insulin, n (%)	4 (10.8)/2 (5.4)
ACEIs/ARBs, n (%)	15 (40.5)
Beta-blockers, n (%)	4 (10.8)
Calcium antagonists, n (%)	6 (16.2)
CPAP, n (%)	6 (16.2)
Oxygen at home, n (%)	15 (40.5)
Laboratory data	
Hemoglobin (g/L)	137 (126–150)
Platelet count (×10^9/L)	246.0 (186.5–301.0)
Glucose (mmol/L)	5.6 (5.1–7.2)
Creatinine (μmol/L)	72.0 (62.0–91.0)

Abbreviations: ACEIs, angiotensin converting enzyme inhibitors; ARBs, angiotensin II receptor blockers; CPAP, continuous positive airway pressure; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; IQR, interquartile range; PPIs, proton pump inhibitors.

Table 3 Characteristics of the acute exacerbations

	N=37
Severity of current exacerbation	
Moderate, n (%)	29 (78.4)
Severe, n (%)	8 (21.6)
Days of worsening symptoms, median (IQR)	4.0 (3.0–7.0)
pH, median (IQR)	7.41 (7.39–7.42)
PaO_2 (mmHg), median (IQR)	69.0 (64.8–74.3)
$PaCO_2$ (mmHg), median (IQR)	44.0 (39.0–50.5)
Positive culture to bacterial infection, n (%)	26 (70.3)
Virus isolation, n (%)	1 (2.7)
Requirements of systemic corticosteroids, n (%)	36 (97.3)
Requirements of antibiotics, n (%)	34 (91.9)
Requirements of hospitalization, n (%)	8 (21.6)

Abbreviation: IQR, interquartile range.

A numerically higher platelet reactivity was seen during exacerbations in the subset of patients with APT, although not achieving statistical significance (PRI: 72.8%±3.1% vs 61.7%±7.5%, p=0.071; Figure 1). Among those receiving aspirin (n=8), a higher collagen-induced platelet aggregation measured with LTA was observed during AECOPD (56.2%±6.1% vs 43.8%±6.4%, p=0.022), whereas the numerical differences did not reach statistical significance with the other assays sensitive to aspirin therapy (data not shown). In the two patients on clopidogrel, no differences in platelet reactivity were observed during exacerbations compared to stability (data not shown).

Systemic inflammatory parameters

The levels of all inflammatory parameters assessed were augmented during AECOPD compared to the stable state (Figure 2): leukocytes (8.8±0.5 vs 7.3±0.4×10^9/L, p=0.001), IL-6 (5.7±0.8 vs 3.0±0.4 pg/mL, p=0.002) and fibrinogen (5.5±0.7 vs 4.7±0.8 g/L, p=0.021).

The change in platelet reactivity measured by VASP was numerically higher in those patients in whom a greater difference in the levels of inflammatory markers between exacerbation and stability was observed. The most evident and almost statistically significant association was found between ΔPRI and Δleukocyte count split into tertiles (lower tertile: −0.5%±3.4%, mid tertile: 7.2%±3.2%, upper tertile: 9.0%±3.2%; p for trend 0.051; Figure 3), while the association was less marked for IL-6 and fibrinogen (p for trend 0.072 and 0.227, respectively; Figure 3).

Discussion

In this investigation, we evaluated the impact of an acute exacerbation on platelet reactivity in COPD patients with a comprehensive panel of assays in order to explore different

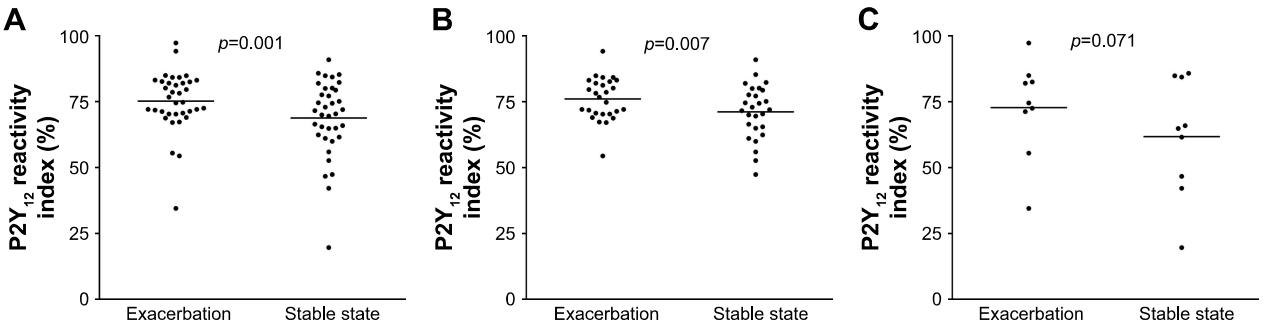

Figure 1 Platelet reactivity measured by VASP assay.
Notes: Black lines represent the least squares mean of the groups. (**A**) Overall population, (**B**) subset of patients without antiplatelet therapy, (**C**) subset of patients under antiplatelet therapy.
Abbreviation: VASP, vasodilator-stimulated phosphoprotein.

platelet signaling pathways. Noteworthy, patients receiving APT were not excluded, expanding upon prior studies by evaluating the impact of these agents on the results. The main findings of this study can be summarized as follows: 1) platelet reactivity is increased during AECOPD; 2) this augmented platelet aggregability is also observed in patients on APT and 3) those patients with greater enhancement of inflammatory markers during an AECOPD are more likely to present a higher increase in platelet reactivity.

Cardiovascular disease is the leading cause of mortality in COPD patients when respiratory failure is excluded.[18] In fact, prior investigations have observed that COPD subjects have an increased risk of ischemic heart disease irrespective of age, gender or smoking status.[19–22] Remarkably, a 2–3-fold increased risk of myocardial infarction has been described during the first 5 days following an AECOPD,[8] which underscores the need for a better understanding of the mechanisms linking AECOPD, inflammation and cardiovascular atherothrombotic complications. Few studies have previously evaluated and observed augmented platelet activation in COPD patients compared to control subjects.[23–26] Further, the impact of an AECOPD in platelet reactivity has been only evaluated in a single experience by Maclay et al who demonstrated increased circulating platelet–monocyte aggregates and, thus, higher platelet reactivity during exacerbations compared to stable COPD.[13] In line with this, our investigation confirmed that exacerbations of COPD lead to a hyperreactive platelet phenotype, showing upregulation of multiple signaling pathways. Noteworthy, this is the first study evaluating the impact of AECOPD on platelet reactivity with platelet function assays with proven ability to predict clinical outcomes in patients with ischemic heart disease.[27]

Table 4 Platelet reactivity during the acute exacerbation and stable state

Test	Overall population, N=37			No antiplatelet therapy, N=27		
	Exacerbation	Stable state	*p*-value	Exacerbation	Stable state	*p*-value
VASP						
PRI (%)	75.2±1.9	68.8±2.4	0.001	76.0±1.5	71.2±2.0	0.007
LTA						
MPA ADP-5 μmol/L (%)	64.2±3.4	60.6±3.6	0.076	68.7±3.5	64.1±4.2	0.069
MPA ADP-20 μmol/L (%)	69.0±2.9	67.4±2.9	0.461	72.5±2.6	69.1±2.8	0.177
MPA AA-1 μmol/L (%)	54.9±6.2	54.0±6.0	0.459	72.8±1.2	71.2±1.7	0.328
MPA Coll-2 μmol/L (%)	72.6±2.5	66.4±3.2	<0.001	78.3±1.3	73.9±1.8	0.009
MEA						
ADP (AU × min)	749.6±41.3	636.4±39.3	<0.001	824.5±33.5	707.6±41.5	0.007
Coll (AU × min)	755.4±40.5	672.51±41.6	0.028	1,049.9±60.5	1,005.3±65.1	0.393
AA (AU × min)	786.0±65.4	731.28±64.9	0.124	948.3±49.0	871.6±48.4	0.067
TRAP (AU × min)	1,048.9±55.8	1,006.3±57.5	0.383	767.4±44.3	648.7±42.7	<0.001

Notes: Platelet reactivity was measured with different platelet function assays in the overall population and in the cohort of patients not receiving antiplatelet therapy. Values are expressed as least squares mean ± SEM. The *p*-values were obtained using repeated-measures analysis of variance.
Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; AU, area under the curve; Coll, collagen; LTA, light transmission aggregometry; MEA, multiple electrode aggregometry; MPA, maximal platelet aggregation; PRI, $P2Y_{12}$ reactivity index; SEM, standard error of the mean; TRAP, thrombin receptor activating peptide; VASP, vasodilator-stimulated phosphoprotein.

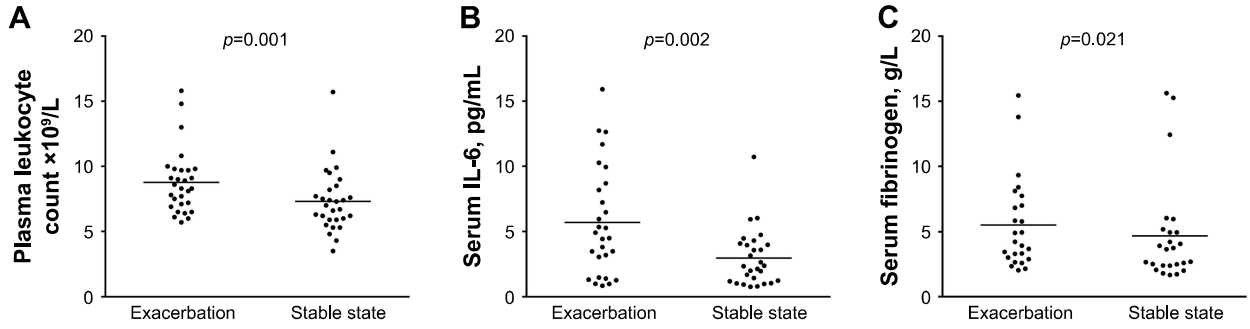

Figure 2 Inflammatory parameters assessed during AECOPD compared to the stable state.
Notes: Black lines represent the least squares mean of the groups. (**A**) Plasma leukocyte count, (**B**) serum IL-6 and (**C**) serum fibrinogen.
Abbreviations: AECOPD, acute exacerbations of chronic obstructive pulmonary disease; IL-6, interleukin-6.

It has been reported that COPD patients suffering a myocardial infarction have worse prognosis than non-COPD patients.[28] This can be partially explained by the fact that COPD patients are less likely to receive guideline-recommended secondary preventive medications with proven benefit, such as antiplatelet agents.[28] Another important feature of our investigation is that patients receiving APT were also included in the study, whereas they were specifically excluded in prior experiences. The finding that patients receiving APT with aspirin or clopidogrel also displayed increased platelet reactivity during an AECOPD raises questions about the efficacy of these two agents in this setting and suggests that a more potent antiplatelet drug could be of help for COPD patients in certain scenarios. Moreover, the results of a recent post hoc analysis of a randomized large-scale trial showed that ticagrelor, a very potent platelet $P2Y_{12}$ inhibitor, was more effective in terms of reducing the risk of ischemic events than clopidogrel (both in addition to aspirin) in COPD patients with an acute coronary syndrome.[29] However, further clinical studies are required to consider if potent antiplatelet treatment must be used during exacerbations in COPD patients with concomitant ischemic cardiovascular diseases.

The exact underlying mechanisms of platelet activation in COPD patients have not been identified, although several factors have been suggested to contribute, such as increased systemic inflammation, hypoxemia and oxidative stress.[30] In particular, COPD is characterized by the presence of low-grade systemic inflammation and an acute inflammatory response during exacerbations of the disease.[7,31] Moreover, the role of inflammation in the formation of atherosclerotic lesions and the occurrence of atherothrombotic complications, where platelets also play a key role, is undisputed.[11,12] In line with this idea, an association between leukocyte count and platelet reactivity has been previously reported.[32] In our investigation, those patients with greater increment of inflammatory markers such as leukocyte count had a numerical trend towards larger increases in platelet reactivity without reaching statistical significance. This finding might support

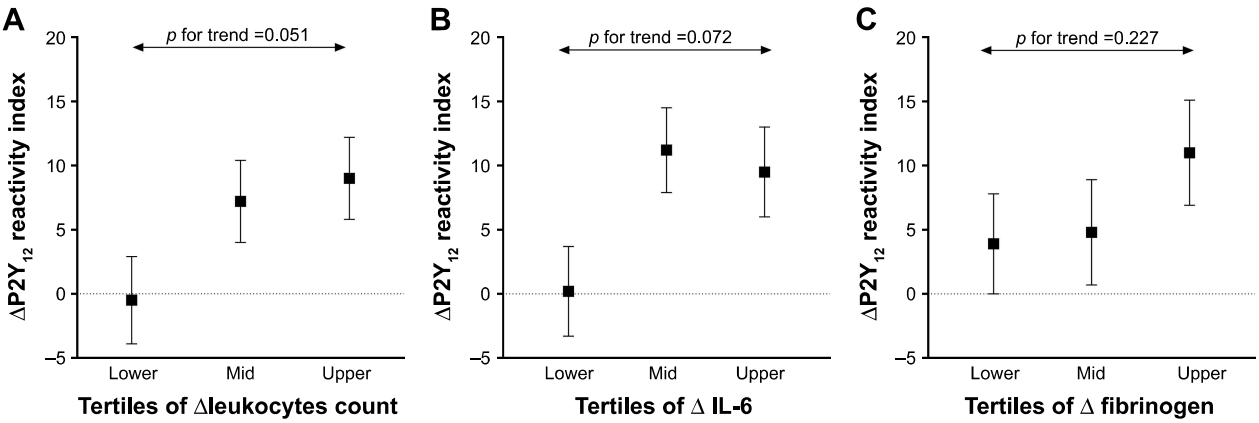

Figure 3 Association between the PRI and the inflammatory parameters.
Notes: The change in PRI was evaluated as the difference: PRI during AECOPD–PRI during stable state. The difference in the inflammatory parameters during AECOPD and stable state was divided into tertiles. (**A**) Difference (Δ) in plasma leukocyte count, (**B**) difference (Δ) in serum IL-6 and (**C**) difference (Δ) in serum fibrinogen.
Abbreviations: AECOPD, acute exacerbations of chronic obstructive pulmonary disease; IL-6, interleukin-6; PRI, $P2Y_{12}$ reactivity index.

the idea of an association between inflammation and platelet activation in COPD patients.

This study has several inherent limitations due to its observational design and relatively small sample size. However, the consistent findings obtained using a variety of assays and agonists are supportive of our study conclusions. The subset of patients receiving APT is particularly small, making the study underpowered to detect differences in this subgroup; therefore, results obtained in this subgroup should be considered exploratory and hypothesis generating. In addition, all patients included in our study had severe and very severe airway limitation, representing an advanced stage of the disease. Therefore, whether the findings of our study can be extrapolated to mild or moderate COPD patients cannot be ascertained.

Conclusion

Platelet reactivity is augmented during acute exacerbations of COPD, irrespective of treatment with antiplatelet agents. This platelet hyperreactivity may contribute to the augmented cardiovascular risk of these patients. In addition, the increase in platelet reactivity could be directly associated with an increment in systemic inflammatory markers during exacerbations.

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Author contributions

MME is the guarantor of the paper, taking responsibility for the integrity of the work as a whole, from inception to the published article. MME and JLF contributed substantially to the study concept and design, data collection, data analysis and interpretation, and the writing of the manuscript. DH, ALM, MLS, GR, JAGH, JD, ÁC and SS contributed substantially to the study design, data analysis and interpretation, and the writing of the manuscript. All authors have read and approved the final version of the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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5. RESUMEN DE RESULTADOS

5.1. Estudio I

Systemic and Pulmonary Vascular Remodelling in Chronic Obstructive Pulmonary Disease. Muñoz-Esquerre M, López-Sánchez M, Escobar I, Huertas D, Penín R, Molina-Molina M, Manresa F, Dorca J, Santos S. PLoSONE. 2016:11(4):e0152987.doi:10.1371/ journal.pone.0152987

Estudio prospectivo realizado en 48 pacientes sometidos a resección pulmonar, divididos en 3 grupos de estudio: 1) 17 pacientes con EPOC, 2) 14 fumadores sin EPOC, 3) 11 nunca fumadores. Se analizaron mediante histomorfometría secciones de la 5ª arteria intercostal y las arterias musculares pulmonares de 100 a 500 micras. Se evaluaron 4 parámetros de engrosamiento intimal: 1) porcentaje del área intimal, 2) porcentaje del estrechamiento luminal, 3) índice de engrosamiento intimal, y 4) relación intima-media. Se analizaron también los factores clínicos asociados a los cambios vasculares y la correlación entre el remodelado de arterias sistémicas y arterias pulmonares.

Los pacientes con EPOC presentan mayor engrosamiento intimal que los fumadores, tanto en las arterias sistémicas (15.6±1.5% vs. 14.2±1.6%, p = 0.038) como en las arterias pulmonares (37.3±2.2% vs. 29.3±2.3%, p = 0.016). Entre los factores clínicos asociados a mayor engrosamiento intimal sistémico, fueron significativos la presencia del síndrome metabólico, el género femenino y la presencia de EPOC. La EPOC fue el único factor clínico asociado al remodelado de arterias pulmonares. Se observó además que existe una correlación entre el engrosamiento intimal de arterias sistémicas y arterias pulmonares (Spearman’s rho = 0.46, p = 0.008).

5.2. Estudio II

Vascular Disease in COPD: Systemic and Pulmonary expression of PARC (Pulmonary and Activation-Regulated Chemokine). Muñoz-Esquerre M, Aliagas E, López-Sánchez M, Escobar I, Huertas D, Penín R, Dorca J, Santos S. PLoS ONE 2017: 12(5): e0177218. doi.org/10.1371/journal.pone.0177218.

Estudio prospectivo realizado en 57 pacientes sometidos a cirugía de resección pulmonar divididos en 3 grupos: 1) 23 pacientes con EPOC, 2) 18 fumadores y 3) 16 nunca fumadores. Se analizó la expresión proteica (Western blot, Inmunohistoquímica e Inmunofluorescencia) y la expresión génica (mRNA) de CCL18/PARC en las arterias sistémicas (intercostales) y arterias musculares pulmonares. Se midieron además los niveles circulantes de CCL18 en todos los pacientes y el grado de remodelado vascular mediante histomorfometría.

Los principales resultados del estudio fueron el hallazgo de un incremento en la expresión proteica de CCL18 en el tejido pulmonar de pacientes con EPOC comparado con el grupo de fumadores (1.96±0.22 vs. 1.29±0.27, P ajustada = 0.038). La proteína CCL18, se localizó predominantemente en la capa de células musculares lisas de las arterias pulmonares musculares. Se observó una correlación significativa entre dicha expresión y el remodelado vascular. No se detectaron diferencias en la expresión génica de CCL18 en ambos tipos de arterias, ni diferencias en el contenido proteico a nivel de las arterias sistémicas en la comparación por grupos. Los niveles circulantes de PARC fueron mayores en los pacientes con EPOC, sin alcanzar la significación estadística.

5.3. Estudio III

Gene and Protein Expression of Fibronectin and Tenascin-C in Lung Samples from COPD Patients. Muñoz-Esquerre M, Huertas D, Escobar I, López-Sánchez M, Penín R, Peinado V, Barberà JA, Molina-Molina M, Manresa F, Dorca J, Santos S. Lung. 2015;193(3):335-343. doi: 10.1007/s00408-015-9717-7.

Estudio prospectivo realizado en 41 pacientes consecutivos sometidos a cirugía de resección pulmonar, divididos en 2 grupos de estudio: 1) 21 fumadores sin EPOC y 2) 20 sujetos con EPOC estable. Se analizó la expresión a nivel pulmonar de las proteínas de la MEC: Fibronectina y Tenascina-C. Se utilizó la técnica de qRT-PCR para el análisis de la expresión génica (mRNA), y técnicas de Western blot e inmunohistoquímica para el análisis de la expresión proteica e inmunolocalización de dichas proteínas en el tejido pulmonar.

Los principales resultados del estudio fueron una mayor expresión génica de Fibronectina en los pacientes con EPOC comparado con los fumadores sin EPOC (4.73 ± 0.54 vs. 2.65 ± 0.57, P = 0.012; respectivamente). No se hallaron diferencias en la expresión génica de Tenascina-C en ambos grupos (EPOC: 2.91 ± 0.44 vs. Fumadores: 2.60 ± 0.48; P = 0.633). A nivel tisular, la expresión pulmonar de FN y TNC se localizó predominantemente en el componente vascular y bronquial, en ambos grupos de estudio y de manera similar.

5.4. Estudio IV

Impact of acute exacerbations on platelet reactivity in COPD patients with and without antiplatelet therapy. Muñoz-Esquerre M, Ferreiro JL, Huertas D, Marcano AL, López-Sánchez M, Roura G, Gómez-Hospital JA, Dorca J, Cequier A, Santos S. International Journal of COPD 2018:13 141–148.

Estudio prospectivo, farmacodinámico ex vivo realizado en 37 pacientes consecutivos con EPOC, donde se analizó la reactividad plaquetar durante la fase de exacerbación y estabilidad. Las pruebas de función plaquetar incluyeron: 1) índice de reactividad $P2Y_{12}$ (PRI) mediante el análisis de la proteína VASP por citometría de flujo, 2) agregometría de electrodos múltiples y 3) agregometría óptica. Se midieron además parámetros de inflamación sistémica como el recuento de leucocitos, niveles séricos de IL-6 y fibrinógeno.

Los principales resultados del estudio fueron que los pacientes con EPOC presentaron una mayor reactividad plaquetar durante las agudizaciones respecto a la fase estable (PRI: 75.2±1.9% vs. 68.8±2.4%, p=0.00; respectivamente). Las otras pruebas de función plaquetar realizadas con diversos agonistas fueron consistentes con los resultados descritos. Los pacientes que recibían terapia antiagregante mostraron también un aumento de la reactividad plaquetar durante la fase aguda aunque ésta no fue estadísticamente significativa (PRI: 72.8±3.1% vs. 61.7±7.5%, p=0.071). Se halló una asociación entre los terciles altos de inflamación (leucocitosis) y el aumento de la reactividad plaquetar durante las agudizaciones, sugiriendo una relación causal.

6. DISCUSIÓN CONJUNTA

La presente tesis doctoral se ha centrado en el estudio de los posibles mecanismos que pueden explicar la relación entre la EPOC y el aumento del riesgo de padecer una enfermedad cardiovascular. En primer lugar, la caracterización e interrelación de las alteraciones vasculares tanto sistémicas como pulmonares así como la expresión de PARC como posible promotor de dichas alteraciones, el análisis de su inmunolocalización y expresión génica, así como los niveles circulantes de dicha proteína (Estudio I y II). En segundo lugar, se enfocó al estudio de la expresión de las proteínas de matriz extracelular FN y TNC en relación al remodelado vascular pulmonar, así como su expresión tanto en el parénquima alveolar como en las estructuras bronquiales (Estudio III). Finalmente, la evaluación mediante pruebas de función plaquetar, del impacto que producen las agudizaciones de la EPOC sobre la agregabilidad plaquetar, con independencia del tratamiento antiagregante recibido (Estudio IV).

6.1 Remodelado vascular y expresión de PARC en la EPOC

Estudios previos han demostrado que el engrosamiento intimal de las arterias sistémicas se debe a la formación de matriz extracelular, lo cual comporta aumento de la secreción y depósito de proteínas, factores de crecimiento y enzimas que regulan la MEC.[94] Este remodelado puede contribuir a diferentes desórdenes vasculares como la arteriosclerosis.[95] Sin embargo, hay que resaltar que la mayoría de estudios diseñados para valorar las alteraciones vasculares se han realizado en tejido arterial procedente de arteriectomías o estudios post-mortem, donde probablemente sólo se incluyan las formas más avanzadas de la enfermedad arteriosclerótica y no los cambios iniciales en la

enfermedad subclínica.[96] Adicionalmente, otros estudios enfocados en demostrar una mayor disfunción endotelial, rigidez arterial o arteriosclerosis subclínica en los pacientes con EPOC utilizan métodos indirectos y no invasivos como por ejemplo los ultrasonidos o la tomografía axial computarizada.[57,97–99] El estudio I de esta tesis fue diseñado específicamente para evaluar estas alteraciones vasculares a nivel histológico, de tal manera que fue posible obtener tejido arterial tanto sistémico (arterias intercostales) como pulmonar del mismo sujeto debido a que se sometía a una cirugía torácica abierta. Los resultados obtenidos permitieron confirmar lo anteriormente descrito, que los pacientes con EPOC presentan un mayor grado de hiperplasia intimal que los fumadores sin EPOC, tanto a nivel pulmonar (donde los cambios son más pronunciados) como a nivel de vasos sistémicos. Este remodelado vascular podría representar las lesiones iniciales de un proceso complejo que progresa a una patología vascular como la arteriosclerosis. Es importante señalar que cuando se analizaron los factores clínicos asociados al remodelado vascular, se encontró que para el caso del remodelado de arterias sistémicas, el género femenino, el síndrome metabólico y la presencia de EPOC eran los principales factores asociados, mientras que para el caso del remodelado vascular pulmonar, sólo la EPOC era relevante. Por tanto, en ambos casos, los resultados obtenidos son consistentes con la hipótesis que en los pacientes con EPOC además de un remodelado vascular propiamente pulmonar, también existe un remodelado vascular sistémico, donde los otros factores de riesgo cardiovascular también juegan un papel importante como se ha descrito ampliamente en la literatura.[100]

En lo que respecta a la interrelación entre ambos territorios vasculares, los resultados de la presente tesis apuntan a una cierta correlación entre el remodelado vascular medido por el porcentaje del engrosamiento intimal a nivel sistémico y pulmonar. En este sentido, investigaciones previas señalan una relación directa entre las lesiones arterioscleróticas de arterias sistémicas y la patología vascular pulmonar evaluada mediante tomografía computarizada y en estudios histológicos post-mortem.[101,102] Estos hallazgos en conjunto refuerzan el concepto de que en ciertas patologías como la EPOC, las alteraciones vasculares sistémicas se producen a la par con las alteraciones pulmonares, donde la inflamación sistémica tendría un rol importante en la promoción de dichos cambios.[103]

El mecanismo supuesto en la presente tesis es que la quimiocina CCL18/PARC juega un papel dentro del proceso inflamatorio causante de las alteraciones vasculares que confieren un mayor riesgo cardiovascular a los pacientes con EPOC. Es por ese motivo que el estudio II se centra en la expresión de CCL18 en ambos territorios vasculares y a nivel circulante. Como resultado principal, se confirma que existe un incremento en la expresión proteica de CCL18 a nivel pulmonar en los pacientes con EPOC, lo cual refuerza la idea de que esta quimiocina podría intervenir en el desarrollo de la enfermedad y ser un marcador de la actividad inflamatoria, como se ha observado en las enfermedades intersticiales pulmonares, en relación a los sitios de inflamación activa. [65,104–107] Adicionalmente, la expresión proteica, evaluada mediante el análisis de inmunomarcaje, nos muestra que a nivel vascular, son las células musculares lisas ubicadas predominantemente en la capa media

arterial, las que expresan CCL18 y que esta expresión se correlaciona con el grado de remodelado vascular (hiperplasia de la capa intima), es decir a mayor remodelado, mayor marcaje y viceversa. Es necesario destacar, que en investigaciones previas realizadas en pacientes con HTP asociada a la EPOC y en HTP primaria, se han descrito cambios fenotípicos de las células musculares lisas, es decir, que son capaces de expresar distintos mediadores, además de producir cambios en el metabolismo celular, más inflamación y proliferación celular.[108–110] Por tanto, esta nueva evidencia, sugiere un posible rol de CCL18 en el remodelado vascular pulmonar que desarrollan los pacientes con EPOC.

Se observa además en este estudio II, que los vasos sistémicos presentan el mismo perfil de expresión de CCL18 que los vasos pulmonares, siendo la capa de CMLs las que expresan esta quimiocina. Sin embargo, con los resultados obtenidos sobre la comparación entre los grupos de estudio no se puede demostrar la importancia de CCL18 en el desarrollo del remodelado sistémico en pacientes con EPOC. Es importante señalar que si la expresión de CCL18 se relaciona a la severidad de las alteraciones vasculares, como se ha descrito previamente a nivel pulmonar, es posible que la expresión sea menor a nivel sistémico por la presencia de un remodelado más discreto, comparado con el pulmonar, lo que explicaría la escasa diferencia entre la expresión de CCL18 en el grupo de pacientes con y sin la enfermedad. De hecho, en estudios histológicos proveniente de pacientes con arteriosclerosis, CCL18 se expresa en las placas de ateroma, asociada a los cambios más extensos y a los focos inflamatorios ricos en macrófagos.[67,68]

Existen indicios de la utilidad de CCL18 como marcador sérico asociado a mortalidad global en 2 grandes cohortes de pacientes con EPOC[69,70] y, por otro lado, como biomarcador asociado al riesgo de ruptura de lesiones aneurismáticas aórticas y de eventos cardiovasculares en pacientes con vasculopatía previa.[111,112] Sin embargo, en el estudio II los niveles séricos de CCL18 no se asociaron al remodelado vascular observado ni sistémico ni pulmonar. Se observó una tendencia de niveles más altos en el grupo de pacientes con EPOC comparado con los controles, la cual no fue estadísticamente significativa, probablemente debido a la variabilidad biológica entre sujetos y al limitado número de casos para ese análisis concreto.

6.2 Expresión génica y proteica de FN y TNC a nivel pulmonar

El remodelado vascular de las arterias pulmonares ocurre desde etapas tempranas de la EPOC y asociado al proceso inflamatorio local [8 p3], por lo que el estudio de los posibles mediadores que contribuyan a perpetuar el proceso inflamatorio, o intervengan en la reparación, reconstrucción y remodelado de los tejidos son de especial interés. En concreto, existe poca evidencia acerca de la expresión de la FN y TNC, dos componentes de la matriz extracelular potencialmente relevantes en estos procesos de remodelado, en la EPOC. Es por ese motivo, que el estudio III describe la expresión pre y post transcripcional de FN y TNC a nivel pulmonar. En primer lugar, se confirma una expresión génica aumentada de FN en los sujetos con EPOC comparada con controles fumadores. Este dato es consistente con un análisis previo donde se describe, que en etapas iniciales de la enfermedad, como es el caso de los sujetos

incluidos en el estudio III, existe una expresión incrementada de FN y a medida que progresa la enfermedad esta expresión génica se reduce.[84] Estos hallazgos refuerzan la idea de que FN podría promover los cambios iniciales tanto del remodelado vascular como de la vía aérea y a medida que progresa la enfermedad, la destrucción de parénquima y la disminución del lecho vascular, esta FN disminuye su expresión. Cabe destacar que dicha expresión génica podría alterarse por la exposición al humo del tabaco, lo cual se ha descrito previamente en estudios *in vitro*, donde las células expuestas al humo del tabaco se inhiben, no proliferan y disminuye la producción de proteínas de la MEC, incluida la FN.[113] En este sentido, en el sub-análisis realizado en sujetos con EPOC y controles según tabaquismo activo o extabaquismo se objetivó una disminución de la expresión génica de FN en los sujetos fumadores activos respecto a los exfumadores.

En segundo lugar, la expresión génica incrementada de TNC a nivel pulmonar en pacientes con EPOC no se ha podido demostrar en el presente estudio. Sin embargo cabe recordar, que esta proteína en principio sólo se expresa durante la embriogénesis y durante la reparación de tejidos; por tanto, no se expresa constitutivamente en el tejido sano de adultos.[76,77] Es decir, los resultados muestran una “re-expresión” a nivel pulmonar tanto en fumadores sin EPOC como en fumadores con EPOC, por lo que no se puede descartar que sea el tabaco, el que induciría la formación de dicha proteína a nivel pulmonar como respuesta a esta injuria. Por otro lado, investigaciones previas realizadas en el contexto de hipertensión pulmonar asociada a EPOC y fibrosis pulmonar, muestran que los genes de la MEC son una de las vías principalmente

expresadas en el tejido vascular remodelado. Sin embargo, la TNC se asocia a la HTP de la fibrosis pulmonar, y no tanto a la HTP de la EPOC.[87] En este campo queda por determinar si el desarrollo de los cambios vasculares en las diversas patologías pulmonares comparten o no una vía patogénica común y el papel que tiene TNC en dicho remodelado.

Finalmente, los resultados obtenidos del análisis de la expresión proteica de FN y TNC en las estructuras pulmonares, concuerdan con las escasas descripciones en patología vascular.[46,83,87] Principalmente FN se expresa en los vasos pulmonares, tanto en la capa endotelial y de CMLs, en células epiteliales bronquiales y en el parénquima alveolar. En cuanto a TNC, se observó principalmente en la capa de CMLs de arterias musculares pulmonares, epitelio bronquial y parénquima alveolar. Sin embargo, no se encontraron diferencias significativas en la comparación por grupos. De hecho, el análisis del contenido proteico de FN y TNC confirma que no hay diferencias importantes entre la expresión de dichas proteínas en fumadores con y sin EPOC, lo que pone de manifiesto la posible interferencia del tabaquismo sobre la expresión de dichas proteínas más allá de la presencia de EPOC.

6.3 Impacto de las agudizaciones sobre la reactividad plaquetar.

La evaluación del impacto de las agudizaciones sobre la reactividad plaquetar de los pacientes con EPOC realizado en el estudio IV, donde se utiliza un panel exhaustivo de pruebas de función plaquetar, pone de manifiesto un mecanismo que podría explicar en parte el riesgo incrementado de eventos cardiovasculares durante las agudizaciones. Concretamente, se observa una

mayor reactividad plaquetar incluso en los sujetos bajo tratamiento antiagregante: aspirina (AAS) o clopidogrel durante la fase aguda respecto a la fase estable de la enfermedad. El presente resultado, unido a la evidencia previa de una mayor activación plaquetaria en pacientes con EPOC[114–116] y al incremento de agregados monocito-plaquetarios circulantes durante las agudizaciones,[93] confirman la hipótesis de que existe un fenotipo de plaqueta hiperreactiva durante las AEPOC. Es de relevancia que no sólo existe una reactividad plaquetar aumentada, sino que ésta se produce a través de múltiples vías de señalización, como sucede en otras enfermedades con riesgo incrementado de eventos cardiovasculares como la diabetes mellitus.[117] Todo ello explicaría lo que se ha observado a nivel epidemiológico, un riesgo incrementado de 2 a 3 veces de padecer un infarto de miocardio durante los primeros 5 días de la agudización de la EPOC.[88]

Otro aspecto que resulta de interés, debido a que no existen datos previos al respecto, es que los sujetos incluidos en el estudio que tomaban AAS o clopidogrel por alguna patología cardiovascular previa, también presentaban un aumento en la agregabilidad plaquetar durante las AEPOC. En este sentido, un reciente subanálisis proveniente de un ensayo clínico a gran escala muestra que ticagrelor, un fármaco muy potente en la inhibición del receptor $P2Y_{12}$, es más efectivo en términos de reducción de riesgo de eventos isquémicos que el clopidogrel (ambos asociados a AAS) en pacientes con EPOC y síndrome coronario agudo.[118] Por tanto, serán necesarios más estudios que evalúen la eficacia de la terapia antiagregante en los pacientes con EPOC y en especial durante las AEPOC.

Finalmente, el mecanismo por el que estas agudizaciones pueden aumentar la agregabilidad de las plaquetas, no está dilucidado. Existen varios fenómenos que ocurren, muchas veces a la par, durante las agudizaciones como son el aumento de la inflamación, la hipoxemia y el aumento del estrés oxidativo.[119] En el estudio IV de la presente tesis, se pudo evidenciar que los pacientes que presentaron durante la agudización una mayor inflamación (terciles medio y superior del incremento de parámetros inflamatorios) presentaron también un mayor aumento en la reactividad plaquetar, lo que confirma en parte lo descrito previamente como una relación directa entre el recuento de leucocitos circulantes y la reactividad plaquetar.[120]

7. CONCLUSIONES

i. Los pacientes con EPOC presentan un mayor remodelado vascular caracterizado por la hiperplasia de la capa íntima de las arterias sistémicas y de las arterias musculares pulmonares, desde las fases iniciales de la enfermedad, lo cual confirma que estos pacientes presentan alteraciones de la pared vascular que podrían conferir un riesgo para desarrollar eventos cardiovasculares.

ii. Existe una correlación entre el remodelado observado en las arterias sistémicas y el remodelado de las arterias musculares pulmonares, es decir, que dichas alteraciones vasculares podrían ser fenómenos que se desarrollan simultáneamente en la EPOC.

iii. Se observa un incremento en la expresión proteica de CCL18 en el tejido pulmonar de los pacientes con EPOC, localizándose principalmente en la capa de células musculares lisas de la pared arterial y asociándose al remodelado vascular, lo que supondría que esta proteína podría tener un papel en el remodelado de las estructuras pulmonares en el contexto de la EPOC.

iv. No se puede demostrar la importancia de CCL18 en el desarrollo del remodelado sistémico en pacientes con EPOC en el presente estudio, debido probablemente a la presencia de un remodelado más discreto comparado con el remodelado de vasos pulmonares.

v. Los sujetos con EPOC presentan un aumento de la regulación génica pulmonar de FN, pero no de TNC comparado con los fumadores.

vi. A nivel tisular, se evidencia una expresión de FN y TNC en las arterias musculares pulmonares y en el epitelio bronquial.

vii. Existe un incremento de la reactividad plaquetar durante las agudizaciones de la EPOC. Este incremento también se observa en sujetos bajo tratamiento con antiagregantes plaquetarios en monoterapia.

viii. Los estudios de función plaquetar que evalúan diferentes vías de señalización celular ponen de manifiesto que durante las agudizaciones de la EPOC existe un fenotipo de plaqueta hiperreactiva.

ix. El proceso inflamatorio desencadenado durante la fase aguda podría ser un mecanismo causal del aumento mostrado en la agregabilidad plaquetar.

Por lo tanto, como conclusión final de esta tesis se puede decir que los pacientes con EPOC presentan alteraciones tanto en la pared vascular sistémica como pulmonar, con expresión de CCL18 como posible factor asociado al remodelado y un aumento de la agregabilidad plaquetar durante las agudizaciones. Todos estos mecanismos podrían estar posiblemente implicados en el aumento del riesgo cardiovascular de estos pacientes.

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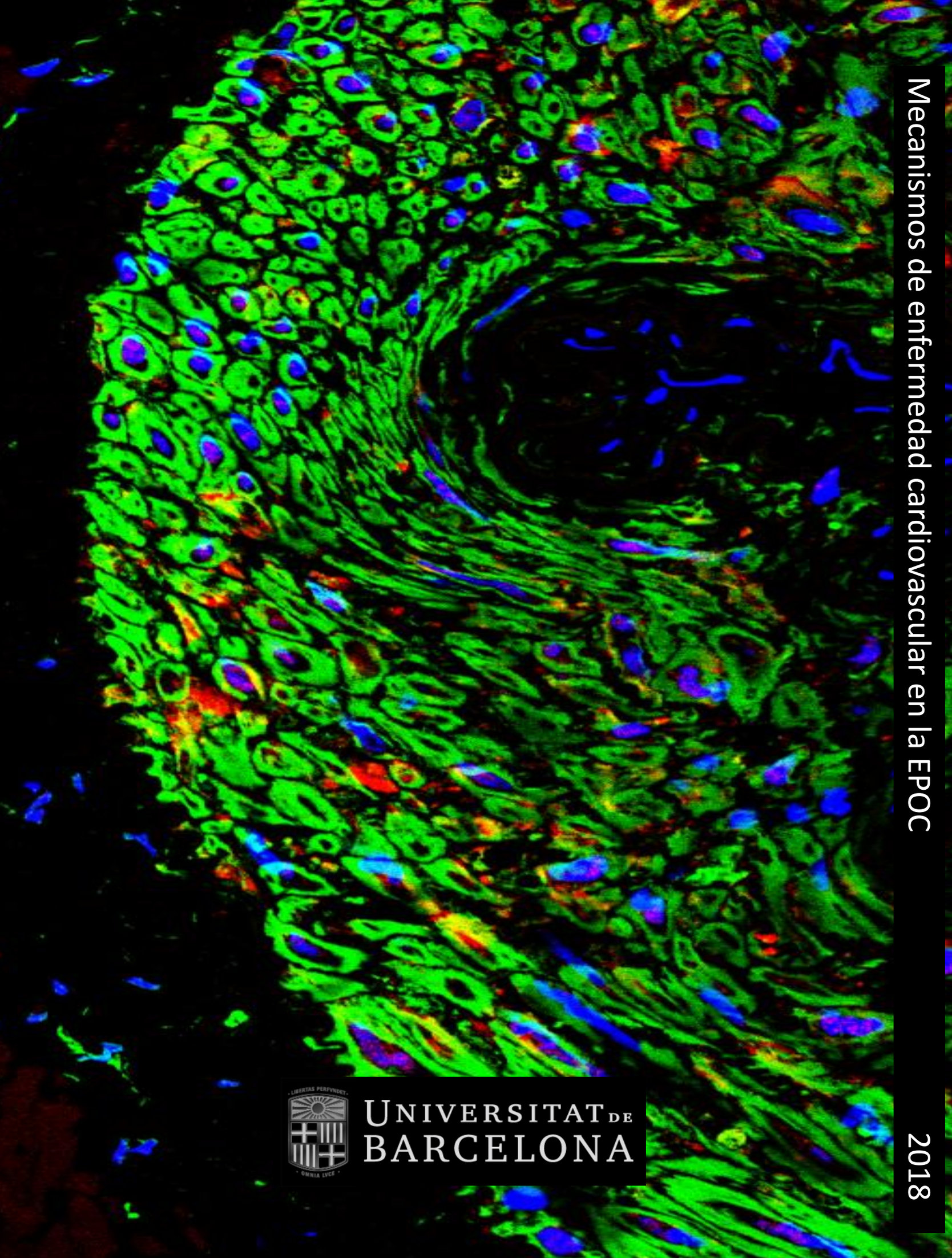

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