Universitat Autònoma de Barcelona

Facultat de Medicina
Departament de Medicina

ESTUDI DE LA COLONITZACIÓ BRONQUIAL BACTERIANA EN EL MALALT AMB MALALTIA PULMONAR OBSTRUCTIVA CRÒNICA ESTABLE: EFECTE SOBRE LA INFLAMACIÓ DE LA VIA AÈRIA I SISTÈMICA

Tesi realitzada per

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Tesi realitzada sota la direcció del Dr. Eduard Monsó i Molas
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CERTIFIQUEN

Que la Tesi Doctoral titulada "Estudi de la colonització bronquial bacteriana en el malalt amb MPOC estable: efecte sobre la inflamació de la via aèria i sistèmica" ha estat realitzada per Alícia Marin Tapia sota la seva direcció, i és apta per a la seva defensa pública davant d'un tribunal per optar al grau de Doctora per la Universitat Autònoma de Barcelona.

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ABREVIATURES

A	adenina
ADN	àcid desoxiribonucleic
ARN	àcid ribonucleic
BAL	rentat broncoalveolar
C	citosina
CVF	capacitat vital forçada
DE	desviació estàndard
ELISA	Enzyme-Linked ImmunoSorbent Assay
G	guanina
h	hores
IC	interval de confiança
IgA	immunoglobulina A
IL	interleuquines
IQR	rang interquartil
mm	mil·límetres
Mm	milimols
ml	mil·lilitre
MPPs	microorganismes potencialment patògens
MPOC	malaltia pulmonar obstructiva crònica
OR	odds ratio
PCR	reacció en cadena de polimerasa
T	timina
TNF-α	factor de necrosis tumoral alfa
UFC	unitats formadores de colònies
VEMS	volum espiratori màxim en el primer segon

RESUM

La Tesi Doctoral titulada "Estudi de la colonització bronquial bacteriana en el malalt amb MPOC estable: efecte sobre la inflamació de la via aèria i sistèmica", es presenta per compendi de publicacions d'articles originals publicats dintre d'una mateixa línia d'investigació.

La memòria es fonamenta en la presentació de quatre treballs científics originals en els quals es reflecteix el resultat de la investigació sobre la colonització bacteriana de l'arbre bronquial en els pacients amb Malaltia Pulmonar Obstructiva Crònica (MPOC) en fase clínica d'estabilitat.

Els articles presentats en aquesta Tesi Doctoral, aporten informació rellevant sobre les característiques microbiològiques de la colonització bronquial i sobre els factors del propi hoste que afavoreixen aquesta colonització, destacant la importància de *Haemophilus influenzae* com a patogen colonitzador per la seva associació amb una major inflamació local i una pitjor qualitat de vida en aquests malalts. Tanmateix, s'observa una associació de la colonització bronquial amb un augment en els marcadors d'inflamació sistèmica com la Proteïna C-reactiva, a nivells que en altres treballs de recerca s'han relacionat amb un major risc per patir accidents cardiovasculars i mort. La magnitud de la inflamació sistèmica associada a la colonització bronquial podria tenir, doncs, efectes sobre l'evolució de la malaltia.

Aquestes troballes confirmen el protagonisme de la colonització bronquial per *Haemophilus influenzae* com a determinant de la inflamació bronquial i sistèmica, potencialment tractable amb l'objectiu d'influir en la prognosi de la malaltia.

En aquesta línia també s'ha investigat per mitjà d'un assaig clínic controlat, l'efecte del tractament amb antibiòtic sobre l'eradicació de la colonització bronquial bacteriana. Els resultats de l'estudi realitzat ajuden a definir el grup de malalts que es podrien beneficiar d'un tractament d'aquestes característiques.

SUBVENCIONS

La doctoranda, Alícia Marin, ha rebut durant la realització d'aquesta tesi beques d'investigació personal adjudicades per la Fundació Catalana de Pneumologia, FUCAP (Beca AstraZeneca any 2004) i per la *Sociedad Española de Neumología y Cirugía Torácica, SEPAR (Beca Becario* any 2004).

L'estudi PAC-EPOC ha estat finançat amb fons procedents del *Fondo de Investigación Sanitaria* (FIS PI060684, FIS PI020541, FIS PI052486, FIS PI052302); Fundació Catalana de Pneumologia; Agència d'Avaluació de Tecnologia i Recerca Mèdiques (AATRM035/20/02); *Sociedad Española de Neumología y Cirugía Toràcica* (SEPAR 2002/137); *Red RESPIRA* (RTIC C03/11); Fundació La Marató de TV3 (num.041110*); CIBERESP y Ciber de Enfermedades Respiratorias-CibeRes* que es una iniciativa del ISCIII.

El treball "Variability and effects of bronchial colonisation in patients with moderate COPD" ha estat finançat per el Fondo de Investigación Sanitaria (FIS PI06084); *Red RESPIRA* (RTIC C03/11); Fundació Catalana de Pneumologia.

Els treballs "Colour of sputum is a marker for bacterial colonisation in chronic obstructive pulmonary disease" i "Efficacy of moxifloxacin in the treatment of bronchial colonisation in COPD", han rebut financiació procedent de Fundació Marató de TV3 i Bayer Schering Pharma.

INTRODUCCIÓ

La malaltia pulmonar obstructiva crònica (MPOC) es defineix com una malaltia respiratòria caracteritzada essencialment per una limitació crònica al flux aeri que no és totalment reversible. Aquesta limitació al flux aeri es sol manifestar en forma de dispnea i, en general, és progressiva. La limitació al flux aeri s'associa a una resposta inflamatòria anormal dels pulmons a partícules nocives i gasos, principalment derivats del fum de tabac, que poden produir altres símptomes com tos crònica, acompanyada o no d'expectoració [1]. Es tracta d'una malaltia infradiagnosticada i amb una elevada morbimortalitat que suposa un problema de salut pública de gran magnitud. Representa un elevat cost sanitari i constitueix la quarta causa de mort a els països del nostre entorn. A més, es preveu que la prevalença de la malaltia continuï augmentant en les properes décades [2].

El curs clínic de la MPOC es crònic, però es caracteritza també per la presència d'episodis d'increment dels símptomes anomenats exacerbacions, significatives en el curs de la malaltia perquè acceleren la progressió de l'obstrucció [3], empitjoren la qualitat de vida [4,5], i són la major causa de morbiditat i mortalitat [6,7]. A més, les exacerbacions contribueixen d'una manera important en l'ús de recursos sanitaris i en la despesa generada per la malaltia [8]. La principal causa d'exacerbacions són les infeccions respiratòries, principalment per bacteris (40-60%), i en segon lloc per virus (30%) [9,10].

El paper dels microorganismes en la patogènesis de la malaltia, tant en la MPOC estable com en les exacerbacions de la MPOC ha estat objecte d'atenció durant moltes dècades. Tot i així encara en queden molts aspectes poc coneguts, com l'efecte de la colonització bronquial en el pacient MPOC estable, els mecanismes de susceptibilitat i el paper dels antibiòtics.

1. Microbiologia de la via respiratòria inferior en el sa i en la MPOC

Els bacteris aïllats a les secrecions respiratòries es divideixen en dos grups: Microorganismes potencialment patògens (MPPs) i microorganismes no potencialment patògens (no-MPPs) [11,12]. Els MPPs son coneguts causants d' infeccions respiratòries. En aquest grup s'inclouen: *Haemophilus influenzae*, *Haemophilus parainfluenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus aureus, Pseudomonas aeruginosa* i diversos membres de la família de les Enterobactèries (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Serratia marcescents* i *Enterobacter cloacae).* Els no-MPPs no causen infeccions respiratòries en individus inmunocompetents, i majoritàriament pertanyen també a la flora normal de la orofaringe i del tracte gastrointestinal. En aquest grup s'inclouen bacteris com *Neisseria* spp., *Enterococcus* spp., *Streptococcus viridans, Corynebacterium* spp., *Staphylococcus* coagulasa-negatius i fongs com *Candida* spp. [11,12].

Determinar la prevalença de bacteris del tracte respiratori inferior ha estat laboriós degut a la dificultat d'obtenir mostres no contaminades per secrecions de la via respiratòria alta. La broncoscòpia amb raspall protegit és el millor mètode per estudiar les secrecions de l'arbre bronquial, ja que evita aquesta contaminació. Mitjançant aquesta tècnica, diversos autors han demostrat una baixa prevalença de colonització per MPPs en el sa respecte el pacient amb MPOC. Rosell i col·laboradors van analitzar els resultats de diferents estudis que utilitzaven la broncoscòpia amb raspall protegit per obtenir mostres de secrecions de la via respiratòria baixa en individus sans i en pacients amb MPOC en estabilitat i en exacerbació, i van trobar que de 70 individus sans només en el 4% es van cultivar MPPs, i això amb càrregues bacterianes baixes [13]. Altres investigadors van confirmar una baixa proporció de MPPs en la via respiratòria baixa d'individus sans [11,12]. Es per això que clàssicament l`arbre bronquial i el parènquima pulmonar han estat considerats estèrils en els subjectes sans.

Contràriament, en els pacients amb MPOC estable freqüentment s'aïllen MPPs, en un percentatge que depèn de la tècnica emprada per recollir la mostra de secrecions. Així, en els estudis que s'utilitzaren mostres d'esput, ja sigui espontani o induït, entre el 38 i el 74% dels cultius van ser positius per MPPs [14-17], mentre que quan s'utilitzaren mostres obtingudes per broncoscòpia i raspall protegit el percentatge fou inferior, entre el 25 i el 31% [12,13,18-20]. Igualment els cultius de les mostres obtingudes per rentat broncoalveolar mostraren un percentatge similar de cultius positius per MPPs, de entre el 33 i el 43% [11,18].

Els MPPs més freqüentment aïllats en els pacients amb MPOC, tant de mostres d'esput com de mostres obtingudes per broncoscòpia, són *Haemophilus influenzae,* en primer lloc, seguit de *Streptococcus pneumoniae i Moraxella catarrhalis* [21,22]*.* A mesura que augmenta la gravetat de la malaltia la flora bacteriana varia cap a un predomini de bacteris Gram-negatius com *Pseudomonas aeruginosa* [23]*.* Amb menys freqüència es troben altres microorganismes com *Staphylococcus aureus* i *Haemophilus parainfluenzae,* encara que el paper d'aquest últim com a patogen és controvertit [22].

2. Efecte de la colonització bacteriana en el pacient amb MPOC estable

La presència d'aquests MPPs a les vies respiratòries baixes en els pacients amb MPOC estable s'ha anomenat colonització bronquial. Aquest terme clàssic que implica que el microorganisme present no té implicacions patològiques, està actualment en revisió doncs, com ja s'ha suggerit en diversos estudis i es confirmarà en aquesta Tesi Doctoral, que la presència d'aquests patògens no és innòcua per l'hoste.

La colonització bronquial es relaciona amb una resposta inflamatòria local i amb un empitjorament del curs de la malaltia. En pacients amb MPOC estable i colonització bronquial s'ha detectat un increment de la cel.lularitat inflamatòria

i dels nivells de marcadors d'inflamació, com són neutròfils, interleuquines 1-β, IL-6, IL-8, IL-10, IL-12, factor de necrosis tumoral alfa, mieloperoxidasa, i leucotriè B4 [11,15,17,18,24]. A més a més, dos estudis han objectivat una relació quantitativa entre la càrrega bacteriana i el nivell de marcadors inflamatoris a l'esput [15,16]. La colonització bronquial pot també contribuir a accelerar la pèrdua de funció pulmonar, com han observat Wilkinson i col·laboradors en un estudi amb pacients amb MPOC estable amb seguiment durant un any, en el qual es va relacionar la pèrdua de VEMS amb un canvi en la microbiologia bronquial paral·lel a un augment de la carrega bacteriana [14]. També s'ha associat la colonització bronquial amb un empitjorament del estat de salut [17], amb un increment de la freqüència de exacerbacions [16,14] i amb inflamació sistèmica [17].

La colonització bronquial es un procés dinàmic amb canvis periòdics en el tipus de patogen colonitzador, canvis en les soques d'un mateix bacteri, i modificacions en la seva càrrega bacteriana al llarg del temps [14,25]. Les diferents espècies bacterianes i també les diferents soques de la mateixa espècie sovint difereixen en virulència i capacitat de produir inflamació [9,15]. En l'estudi de Hill i col·laboradors *Pseudomonas aeruginosa* va ser el bacteri que va demostrar més potencia inflamatòria seguit de *Haemophilus influenzae*, al contrari que *Moraxella catarrhalis* que va mostrar menys capacitat inflamatòria [15]. Contrariament, en un altre estudi Sethi i col·laboradors van trobar que els patògens que produïen mes inflamació eren *Haemophilus influenzae,* i també *Moraxella catarrhalis* [9].

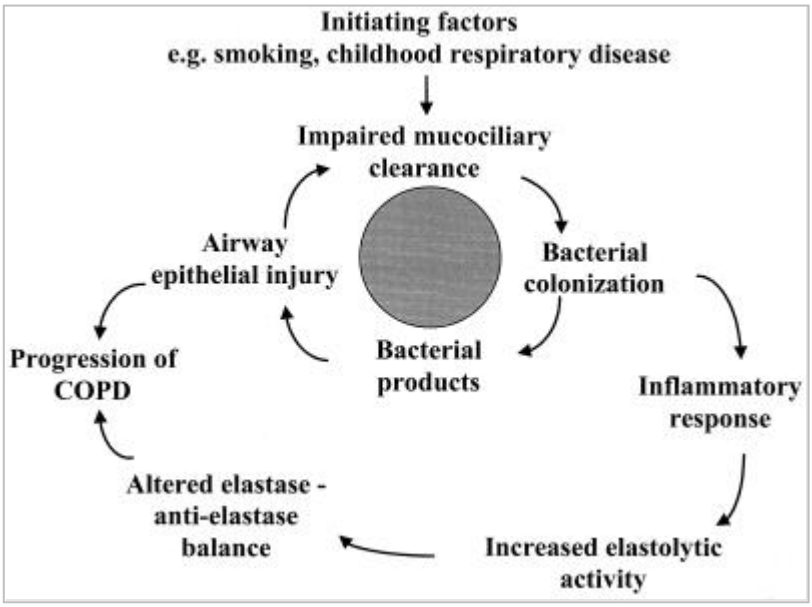
3. Susceptibilitat a la colonització bronquial

En els individus sans el manteniment de la via respiratòria inferior lliure de patògens depèn principalment de l'eficiència del sistema defensiu innat del pulmó. Aquest sistema és multifactorial e inclou les cèl·lules epitelials i l'aclariment mucociliar, els pèptids antimicrobians, la resposta immune local de

la IgA secretora i una resposta immune adquirida. En el pacient amb MPOC s'observen alteracions d'aquest sistema defensiu innat, essent el més important l'alteració de l'aclariment mucociliar produït per l'efecte del tabac que afavoreix el creixement bacterià [26].

En la Hipòtesi del Cercle Viciós, recentment reformulada per autors com Sethi i Murphy [27], postula l'existència d'un cercle viciós entre colonització, infecció i inflamació a la MPOC, segons el qual una disminució en l'eficàcia de la defensa innata dels pulmó en més d'un del seus components afavoreix l'establiment dels patògens a la via respiratòria inferior. La presència d'aquests patògens provoca la cronicitat dels episodis de colonització i l'aparició recurrent d'infecció bronquial, ambdós fenòmens causants d'una resposta inflamatòria persistent que contribueix a una progressió de la malaltia (Figura 1). En aquesta hipòtesi, no obstant, l'evidència definitiva de la relació directa entre progressió de la MPOC i inflamació induïda per la colonització bronquial i les infeccions recurrents està encara per demostrar.

Figura 1. Diagrama representatiu de la Hipòtesi del Cercle Viciós (modificat de Sethi i Murphy [27])



En la línia proposada per la Hipòtesi del Cercle Viciós, un estudi recent de Millares i col·laboradors ha demostrat que els malalts de MPOC colonitzats per *Haemophulis influenzae* tenen nivells inferiors d'IgA específica contra aquest microorganisme i nivells superiors de la forma activa de la metaloproteinasa-9 que els malalts no colonitzats. Així, la colonització per aquest microorganisme pot veure's facilitada per aquest defecte de resposta innata protectora, i causar canvis estructurals en la matriu extracel·lular mitjançant la estimulació de l'activitat de la proteasa [28].

Pel que fa als factors de risc per colonització bronquial més freqüentment identificats a la literatura trobem el tabaquisme actual o passat i la reducció del VEMS i/o CVF [20,29].

4. Antibiòtics en el pacient amb MPOC estable

El reconeixement del potencial paper dels bacteris en el manteniment de la inflamació bronquial en el pacient amb MPOC en fase estable ha portat a plantejar la utilitat del tractament amb antibiòtics de la colonització bacteriana, en estabilitat. Aquet plantejament no és nou i ja en la dècada dels anys 60 es van portar a terme els primers assajos clínics amb antibioteràpia profilàctica en la MPOC. Una meta anàlisi de 9 estudis realitzats amb aquest objectiu va mostrar algun benefici en la reducció de la freqüència de les exacerbacions que no arribava a la significació estadística [30].

Més recentment, i basant-se en experiències prèvies en l'ús de macròlids en altres malalties respiratòries, Seemungal i col·laboradors van analitzar l'efecte de la eritromicina administrada a pacients amb MPOC durant un any i van observar una reducció de les exacerbacions en el grup tractat [31]. Un altre estudi en pacients amb MPOC utilitzant azitromicina 250 mg al dia durant un any va demostrar una reducció en les aguditzacions i una millora de la qualitat de vida en el braç de tractament [32]. Dissortadament, l'ús d'azitromicina es va

associar amb empitjorament de l'audició i amb més resistències a macròlids en els patògens aïllats a les mostres de secrecions nasofaríngies. El risc de desenvolupar resistències es podria veure reduït, però, si els antibiòtics són utilitzats en tandes de curta durada periòdicament, una hipòtesi que no ha estat suficientment estudiada fins aquest moment.

HIPÒTESI DE TREBALL

La colonització bronquial de la via aèria inferior en els malalts amb MPOC en fase d'estabilitat clínica no és innòcua ja que s'associa a un augment de la inflamació de la via aèria i sistèmica, a un nivell suficientment elevat per tenir implicacions en el deteriorament clínic i funcional d'aquests malalts.

OBJECTIUS

Els objectius plantejats en aquesta Tesi Doctoral són els següents:

1. Determinar la prevalença i les característiques de la colonització bronquial en els pacients amb MPOC en fase d'estabilitat clínica.
2. Identificar els factors de risc per colonització bronquial en pacients MPOC en fase d'estabilitat clínica.
3. Investigar la associació entre la colonització bronquial i la inflamació local i sistèmica.
4. Determinar la repercussió de la colonització bronquial sobre la qualitat de vida a la MPOC.
5. Investigar la persistència i la recurrència de colonització bronquial en el temps, així com l'efecte del tractament antibiòtic sobre aquestes.

METODOLOGIA

1. Metodologia general

1.1. Disseny de l'estudi

Els estudis que han conformat el treballs d'aquesta Tesi Doctoral s'han efectuat en pacients amb MPOC examinats en situació d'estabilitat. Els dissenys utilitzats han estat diferents en els quatre estudis que conformen la Tesi. En el primer treball s'ha efectuat un estudi observacional en un cohort, en el segon i el tercer, anàlisis transversals d'una mostra poblacional, i en el quart treball un assaig clínic controlat. Els dissenys específics es detallen a la descripció de cadascun dels treballs.

1.2. Criteris d'inclusió

Tots els pacients estudiats eren adults de >40 anys, fumadors o ex fumadors de, almenys, 10 paquets acumulats/any, amb diagnòstic de MPOC definit per un quocient post broncodilatador entre el volum espirat màxim en el primer segon (VEMS) i la capacitat vital forçada (CVF) inferior o igual a 0.70 i una prova broncodilatadora negativa (increment del VEMS <200 ml i <12% respecte al basal). En el moment de les exploracions, els pacients es trobaven en període d'estabilitat clínica definida com a absència de símptomes d'exacerbació o ús d'antibiòtics o esteroides orals en les 8 setmanes anteriors.

1.3. Criteris d'exclusió

Es van excloure aquells pacients majors de 40 anys, amb diagnòstic previ d'asma bronquial, bronquièctasis o altra patologia pulmonar rellevant diferent de la MPOC.

1.4. Obtenció de l'esput

En tots els casos la mostra d'esput ha estat obtinguda al matí, i processada en els 60 minuts immediats. Quan el pacient no era capaç d'obtenir una mostra d'esput espontani o aquesta mostra era inferior a 1 ml es procedia a la inducció de l'esput. El procediment d'inducció implicava tractar el pacient amb un beta adrenèrgic inhalat deu minuts abans de la nebulització de concentracions creixents de sèrum salí (0.7, 3, 4 y 5%), cadascuna d'elles durant 7 minuts. Després de cada inducció s'intentava l'obtenció d'esput amb la tos, i, si la tos era productiva, es recollia la mostra en un contenidor estèril. Abans de cada nebulització es realitzava una nova espirometria forçada, i el procediment era interromput quan s'objectivava un descens en el VEMS superior al 20% del valor basal. Una part de la mostra d'esput obtinguda es destinava a l'examen microbiològic, i la resta a l'examen dels marcadors d' inflamació.

1.5. Examen microbiològic de l'esput

La primera part de la mostra d'esput obtinguda va ser separada de la saliva contaminant mitjançant examen macroscòpic i va ser pesada i processada amb un volum igual de solució fresca de dithiotreitol (Sputosol, Unipath). Després es va cultivar i es va determinar la càrrega bacteriana mesurada en unitats formadores de colònies/ml (ufc/ml) de la mostra. El protocol de processament microbiològic de la mostra d'esput va ser el següent: a) grau de Murray-Washington, b) càrrega bacteriana, amb preparació de dilucions seriades i cultiu, c) identificació dels microorganismes (morfologia colonial, tinció de Gram, prova d'oxidasa i catalasa, requeriment de factors de creixement, prova de les porfirines, proba API NH, prova de la betalactamasa, tipatge capsular), d) congelació d'un mínim de tres unitats formadores de colònies per a posterior tipatge molecular. En pacients amb cultius polimicrobians es va considerar en l'anàlisi quantitatiu el microorganisme que presentava major càrrega bacteriana.

Els cultius van ser considerats positius per colonització bronquial quan hi creixien microorganismes potencialment patògens (MPPs) com a *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Streptococcus pneumoniae*, *Moraxella catharralis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, o enterobacteries amb càrregues bacterianes de, com a mínim, 100 ufc/ml [12].

1.6. Marcadors d'inflamació i recompte cel·lular de l'esput

Per la determinació de marcadors d'inflamació al sobrenedant, l'esput va ser separat de la saliva contaminant mitjançant examen macroscòpic. La mostra d'esput va ser també pesada i processada amb 4 vegades el seu volum de solució fresca de dithiotreitol, es va homogeneïtzar durant 20 minuts, es va col·locar en el mateix volum de solució de PBS, vortexar durant 15 segons i filtrar a través d'una gasa de niló de 48 micròmetres. Es va utilitzar un hemocitòmetre Neubauer per determinar la viabilitat cel·lular mitjançant exclusió per blau de tripà, i finalment es va centrifugar. El sobrenedant es va congelar i es va utilitzar posteriorment per al mesurament d'interleuquines (IL-1 beta, IL-6, IL-8, IL-10, IL-12) i TNF alfa (cytoquine bead array, BD Biosciences).

Es va determinar el recompte cel·lular absolut i percentual. Es va calcular el total de cèl·lules inflamatòries expressat en número absolut de cèl·lules per gram d'esput, restant les cèl·lules escatoses del total de cèl·lules. Es va calcular el recompte absolut i diferencial per neutròfils, limfòcits i eosinòfils, resultant 400 cèl·lules no escatoses per extensió de Writh.

1.7. Determinació del tipatge molecular

El tipatge molecular microbiològic de les mostres obtingudes es va realitzar per electroforesi en camp polsant. L'extracció de DNA cromosòmic es va realitzar en blocs d'agarosa [33], a partir de microorganismes cultivats en agar xocolata 24-48 h a 36ºC. Breument, els blocs d'agarosa es van preparar barrejant una suspensió de bacteris en tampó Pett IV (10 mM Tris-HCl pH 7.5, 1 M NaCl) amb

Incert agarosa al 2% (Incert, FMC Bioproducts) en tampó EC (6 mM Tris-HCl pH 7.5, 1 M NaCl, 10 mM EDTA, 0,5% Brij 58, 0,2 % Deoxycolat sódic, 0,5 % N-Lauroylsarcosine, 1% Tween). Els blocs es van incubar a 37ºC durant 18 h en solució de Lysis (3mg/ml lisozima Sigma, 0.05 mg/mL RNAsa A, 10mM Tris-HCl pH 7.5, 50 mM EDTA, 0,5% Nonidet P-40, 0,5 % Trinton X-100). Posteriorment, els blocs es tractaren amb proteïnasa K (1mg/ml) en tampó TE (15mM Tris-HCl pH 7.5, 50mM EDTA, 1% Nonidet P-40) durant 18 h a 50 ºC. Per la digestió enzimàtica, els blocs d'agarosa es van equilibrar amb el tampó de digestió durant 1 hora a 4ºC abans d'incubació amb 30 U de l'enzim de restricció Spe I o Sma I segons el necessari (New England Biolabs), durant 18 h a 30ºC. Els fragments de restricció es van separar en un gel d'agarosa al 1% en 0,5x Tris-Borat-EDTA (Sigma-Aldrich) per electroforesi en camp polsant amb un aparell *countour-clamped* de camp elèctric homogeni (CHEF DR II system, Biorad). EL pols inicial va ésser de 5,6 s, que s'augmentava linealment fins un pols final de 40,6 s durant 24 hores a 5 V/cm i a 14ºC. El concatàmer del bacteriòfag lambda (New England Biolabs) es va incloure com marcador de pes molecular de l'ADN. El gel es va tenyir amb bromur d'etidi i es va fotografiar sota un transil·luminador a 360 nm. Els patrons de restricció produïts por l'electroforesi en camp polsant es van analitzar amb el "Diversity Database Sofware" (BioRad) per generar dendogrames.

1.8. Detecció d'ADN de microorganisme per reacció en cadena de la polimerasa (PCR)

L'extracció d'ADN es va realitzar de les mostres de sobrenedant de esput amb cultius negatius per investigar la presència de MPPs. Per a l'extracció d'ADN es va utilitzar QIAamp DNA mini kit (QUIAGEN, Hilden, Alemanya). Breument, es van dissenyar dos parells de encebadors específics per al gen p6 de *Haemophilus influenzae* i *Haemophilus parainfluenzae* utilitzant el software Primer Express (Applied Biosystems, Foster City, California, USA), i per *Moraxella catarrhalis* es van utilitzar els primers descrits prèviament per Greiner i col.laboradors [34]. Les seqüències van ser, encebador directe 5' -

AATTTCCAGCTTGGTCTCCA-3' i invers 5' - CAAAAGTTTGAGCAGCACCA-3, per *Haemophilus. Influenzae*; encebador directe 5'-CCGTTTACTTCGGTTTCGAC-3' i invers 5' - CAGCACGACGTTGACCTAAA-3' per *Haemophilus parainfluenzae* i, encebador directe 5'-GTGAGTGCCGCTTTTATAACC-3 ' i invers 5'-TGTATCGCCTGCCAAGACAA-3 ' per a *Moraxella Catarrhalis*.

Un total de 10 µl del producte de PCR es va fer fluir a través d'un gel d'agarosa al 2% en TBE i es va visualitzar amb bromur d'etidi. Les mides dels productes amplificats, 167 bp, 171 bp i 71 bp, per a *Haemophilus influenzae*, *Haemophilus parainfluenzae* i *Moraxella catarrhalis* respectivament, es van comparar amb un control positiu i amb un marcador de pes molecular (Promega, Madison, WI, UE). Les mostres que van mostrar ADN de MPP amb un cultiu positiu posterior per al mateix MPP en els següents 3 mesos es van considerar com colonitzats per el MPP, i el resultat negatiu del cultiu de l'esput en la mostra corresponent es va considerar com a fals negatiu.

2. Metodologia específica

2.1. Estudi 1: *Variability and effects of bronchial colonisation in patients with moderate COPD.*

Treball de tipus prospectiu amb un disseny de cohort en el que es van incloure de manera consecutiva pacients amb MPOC procedents de la consulta externa de Pneumologia d'un hospital terciari de l'àrea d'influència de Barcelona en estabilitat clínica en els últims dos mesos. Els pacients van ser examinats en el moment del reclutament i als 9 mesos del mateix.

2.2. Estudi 2: *Effect of bronchial colonisation on airway and systemic inflammation in stable COPD*

Treball de tipus transversal en el qual es van analitzar dades basals d'una part dels pacients inclosos a la Cohort PAC-MPOC (Fenotip i curs de la Malaltia

Pulmonar Obstructiva Crònica). Aquesta cohort estava formada per pacients reclutats en 9 hospitals terciaris espanyols durant un primer ingrés hospitalari per una agudització de MPOC i examinats com a moment basal als tres mesos en estabilitat.

2.3. **Estudi 3:** *Colour of sputum is a marker for bacterial colonisation in chronic obstructive pulmonary disease*

Treball de tipus tranversal en el qual es van analitzar les característiques clíniques associades amb la colonització bronquial en pacients amb MPOC estable els quals van ser consecutivament reclutats durant un any en la consulta externa de dos hospitals terciaris de l'àrea d'influència de Barcelona.

2.4. **Estudi 4:** *Efficacy of moxifloxacin in the treatment of bronchial colonisation in COPD*

Assaig clínic, prospectiu, aleatori, doble cec i controlat amb placebo que es porta a terme en els serveis de Pneumologia de 2 centres hospitalaris de l'àrea d'influència de Barcelona en pacients amb MPOC estable i colonització bronquial de la via aèria inferior, els quals van ser consecutivament reclutats durant un any en la consulta externa. Els pacients van ser aleatoritzats 1:1 a rebre placebo o moxifloxací.

PUBLICACIONS

A continuació es detallen els articles originals publicats que fonamenten aquesta Tesi Doctoral

1. **Estudi 1:** *Variability and effects of bronchial colonisation in patients with moderate COPD.*

A.Marín, E.Monsó, M.García-Núñez, J.Sauleda, A.Noguera, J.Pons, A.Agustí and J.Morera.

Eur Resp J 2010; 35: 295-302

Factor d'impacte 2008: 5.922

Resum

La finalitat d'aquest estudi és determinar la prevalença de colonització bronquial en una cohort de pacients amb MPOC de grau moderat en fase d'estabilitat clínica i la relació entre les característiques d'aquesta colonització amb marcadors d'inflamació bronquial i canvis en el VEMS post broncodilatador durant el seguiment.

Es varen recollir mostres d'esput en situació d'estabilitat clínica per anàlisi microbiològic i de marcadors d'inflamació bronquial (neutrofília, TNF-α, interleuquines IL-1β, IL-6, IL-8, IL-10, IL-12) així com proves de funció pulmonar en una visita a l'inici de l'estudi i en una segona ocasió als 9 mesos de seguiment.
Els microorganismes periòdicament aïllats en el mateix pacient que varen mostrar el mateix perfil molecular utilitzant la tècnica de electroforesis de camp polsant es van considerar com a colonització persistent.

Es va observar colonització bronquial en 56 de les 79 mostres recollides (70.9%), essent els patògens més freqüentment aïllats *Haemophilus influenzae, Pseudomonas aeruginosa* i enterobactèries (n=47). La colonització per tots aquests patògens es va associar amb neutrofília a l'esput ($p<0.05$, Test de Chi-Quadrat). La resposta inflamatòria neutrofílica bronquial es va associar estadísticament de manera significativa amb una pèrdua de VEMS durant el seguiment (OR 2.67, IC 95% 1.07-6.62).

Haemophilus influenzae, a més a més, s'associà a nivells elevats de IL-1β ($p=0.005$) i IL-12 ($p=0.01$), amb una relació dosi-resposta (Coeficient de correlació de Spearman de 0.38 per IL-1β ($P=0.001$), i de 0.32 per IL-12 ($p=0.006$). Es va observar persistència de la mateixa soca bacteriana al llarg del temps en 12 ocasions (21.4%), fonamentalment quan el MPP era *Pseudomonas aeruginosa* o una enterobactèria. *Haemophilus parainfluenzae* no presentava associació amb cap resposta inflamatòria.

Resultats

40 pacients amb MPOC en fase d'estabilitat clínica varen complir els criteris d'inclusió. Els pacients inclosos eren quasi tots del sexe masculí (39, 97.5%), amb una edat mitjana±DE de 66.5±8.1 anys i amb una alteració moderada de la funció pulmonar (VEMS post broncodilatador 58% del predit (DE 19%). El temps de seguiment va ser de 8±3.2 mesos i es van realitzar 79 visites en total.
Es va observar colonització bronquial en 56 de les 79 mostres recollides durant el seguiment (70.9%), precedides per 42 mostres amb cultiu positiu a la visita basal. Aquesta colonització bronquial observada en el seguiment era deguda principalment a l'adquisició d'una nova soca bacteriana (30, 38%). La colonització persistent per la mateixa soca bacteriana només es va observar en 12 casos (15%), principalment per *Pseudomonas aeruginosa* o enterobactèries. No es van trobar diferències estadísticament significatives en els paràmetres inflamatoris mesurats entre les mostres amb soques de nova adquisició o soques persistents.

Haemophilus influenzae va ser el patogen més freqüentment aïllat en mostres en quasi tots els casos amb càrregues bacterianes elevades. La identificació d'aquet microorganisme es va associar amb una major resposta inflamatòria bronquial mesurada a l'esput per nivells elevats de IL-1β i IL-12 i neutrofília, amb una correlació positiva entre la càrrega bacteriana i la IL-1β. També les mostres colonitzades per *Pseudomonas aeruginosa* i Enterobactèries varen mostrar una resposta neutrofílica elevada, encara que no un augment de Interleuquines. Aquesta resposta neutrofílica durant el seguiment estava associada significativament amb un empitjorament de la funció pulmonar mesurada per la pèrdua de FEV1., després d'ajustar per altres variables.
No es va trobar cap resposta inflamatòria en aquelles mostres colonitzades per *Haemophilus parainfluenzae.*

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Variability and effects of bronchial colonisation in patients with moderate COPD

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ABSTRACT: Sputum and lung function were periodically assessed in stable moderate chronic obstructive pulmonary disease (COPD) outpatients to determine relationships between bronchial colonisation and inflammation.

Relationships between potentially pathogenic microorganism (PPM) typology, bronchial inflammation (neutrophilia, tumour necrosis factor-α, interleukin (IL)-1β, IL-6, IL-8, IL-10 and IL-12) and post-bronchodilator decline in forced expiratory volume in 1 s (FEV_1) were analysed. PPMs periodically showing the same molecular profile using pulse field gel electrophoresis were considered long-term persistent.

Bronchial colonisation was observed in 56 out of 79 follow-up examinations (70.9%) and was mainly due to *Haemophilus influenzae*, *Pseudomonas aeruginosa* and enterobacteria (n=47). These PPMs were all related to sputum neutrophilia ($p \leqslant 0.05$, Chi-squared test), and *H. influenzae* was related to higher levels of IL-1β ($p=0.005$) and IL-12 ($p=0.01$), with a dose–response relationship (Spearman's correlation coefficient of 0.38 for IL-1β ($p=0.001$), and of 0.32 for IL-12 ($p=0.006$)). *Haemophilus parainfluenzae* was not associated with an identifiable inflammatory response. Long-term persistence of the same strain was observed in 12 examinations (21.4%), mainly due to *P. aeruginosa* or enterobacteria. A neutrophilic bronchial inflammatory response was associated with a statistically significant decline in FEV_1 during follow-up (OR 2.67, 95% CI 1.07–6.62).

A load-related relationship to bronchial inflammation in moderate COPD was observed for colonisation by *H. influenzae*, but not for colonisation by *H. parainfluenzae*.

KEYWORDS: Bronchial colonisation, chronic obstructive pulmonary disease, *Haemophilus influenzae*, interleukin-1β, interleukin-12, potentially pathogenic microorganisms

The bronchial tree and the pulmonary parenchyma are sterile in healthy subjects, but in patients with chronic obstructive pulmonary disease (COPD) potentially pathogenic microorganisms (PPMs) are often recovered from bronchial secretions during periods of clinical stability and, particularly, during episodes of exacerbation, when PPM loads increase significantly [1, 2]. Most studies on PPM colonisation in COPD have focused on patients with severe disease [3–5]. In contrast, the relationships between the microbiology of bronchial colonisation and local inflammatory response in moderate COPD patients, when therapeutic interventions are expected to have maximal effects, have only been assessed in small selected population samples [6–8]. With the hypothesis that the characteristics of bronchial colonisation have an effect on bronchial inflammation and lung function that may be identified in stable outpatients with moderate COPD, the present study sought: first, to determine the prevalence and load of bronchial colonisation in a cohort of COPD outpatients with moderate airflow limitation never admitted to a hospital for an exacerbation of the disease; and secondly, to investigate in these patients the relationships between colonisation characteristics, bronchial inflammation markers and post-bronchodilator changes in forced expiratory volume in 1 s (FEV_1) during follow-up.

METHODS

Design and study population

We enrolled a cohort of stable COPD outpatients diagnosed according to the criteria of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) [9] and reporting no admissions for respiratory symptoms. Patients were examined at

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baseline in stable condition from ⩾8 weeks, and their sociodemographic data, smoking habits, respiratory symptoms, treatments, sputum microbiological and inflammatory characteristics, and lung function were recorded. 9 months (interval 7–11 months) after this baseline assessment, a follow-up visit was scheduled at a time when the patient had been in a stable condition for ⩾8 weeks. That visit included a second assessment of sputum characteristics, together with recording of exacerbation history and assessment of lung function changes since the previous visit. Patients were excluded if they: were <40 yrs of age; had ever been admitted to a hospital for respiratory symptoms; were diagnosed with asthma, cystic fibrosis, neoplasia or bronchiectasis, and/or were being treated with oral corticosteroids or immunosuppressors for any reason. Additionally, patients who did not maintain the same smoking habits and baseline bronchodilator and inhaled corticosteroid treatment throughout the follow-up interval were excluded from analysis. Acute episodes of increased dyspnoea, sputum production and/or purulence appearing during follow-up and treated with antibiotics and/or oral corticosteroids by a physician were considered as exacerbations [10, 11]. The present study was reviewed and approved by the local research ethics committee in Catalonia, Spain.

Interview questionnaire and lung function

The interview questionnaire, which included items on age, sex and chronic bronchitis (defined as cough and phlegm >3 months each yr) was obtained from the European Community Respiratory Health Survey (ECRHS) [12, 13]. Current treatments and previous exacerbations were also recorded. All subjects performed forced spirometry and reversibility tests in the morning with the same dry rolling-seal spirometer (Spirometrics, Gray, ME, USA) at baseline and follow-up visits according to standard techniques [14]. Forced vital capacity and FEV_1 were measured and were compared with age- and height-adjusted reference values obtained from selected volunteers from the Barcelona province [15]. This was followed by a reversibility test with salbutamol. Results were expressed as absolute values (mL) and percentages of the reference values.

Sputum sampling and analysis

An induced sputum sample was obtained and processed within 60 min at each visit according to standard methods [16, 17]. In brief, the patient was pre-treated with an inhaled β_2-agonist 10 min before nebulisation of isotonic saline (0.9%); this was followed by increasing concentrations of hypertonic saline (3, 4 and 5%) for 7 min with each concentration. After each induction the patient attempted to obtain a sputum sample by coughing, and the nebulisation procedure was halted when the sputum volume collected was ⩾1 mL [18]. Sputum induction was performed after 6 h of abstinence in current smokers. Samples with >25 leukocytes per field (Murray-Washington ⩾3) were considered indicative of a neutrophilic inflammatory response [19, 20].

Microbiological determinants

The sputum sample was weighed and processed with an equal volume of dithiothreitol (Sputasol; Oxoid Ltd, Basingstoke, UK), cultured, and the microbial load of the sample was determined [21]. The determination of microbial typology and load was carried out by means of serial dilutions and culture in selective media, according to standard methods [22], with quantitative cultures expressed as colony-forming units (cfu)·mL^{-1}. For the purposes of this study, cultures were considered positive for bronchial colonisation according to previously defined criteria [23–25] if they grew microorganisms, at loads of ⩾100 cfu·mL^{-1}, that were considered as potentially pathogenic, such as *Haemophilus influenzae, Haemophilus parainfluenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Pseudomonas aeruginosa*, enterobacteria and/or *Staphylococcus aureus*.

Molecular genotyping

Molecular PPM typing was performed using pulse field gel electrophoresis (PFGE) to determine whether a PPM recovered from consecutive samples corresponded to the persistence of the same strain or to the acquisition of a new one [26]. In brief, chromosomal DNA from multiple (more than four) PPMs growing in chocolate agar was extracted with agarose and incubated at 37°C for 18 h. After enzyme digestion with Sma I (New England Biolabs, Ipswich, MA, USA), restriction fragments were separated in agarose gel with tris-borate-ethylenediaminetetraacetic acid (Sigma Chemical, St Louis, MI, USA) through PFGE using a homogeneous electric camp contour clamp (CHEF DR II system; BioRad, Ivey sur Seine, France), beginning with an initial 5.6-s pulse that was linearly increased until a 40.6-s pulse was reached and then maintained for 24 h at 5 V·cm^{-1} and 14°C. Bacteriophage λ concatemer (New England Biolabs) was included as the molecular weight DNA marker. Obtained patterns were photographed with a 360-nm transilluminator after staining, and analysed using Diversity Database Software (BioRad). PPMs cultured from follow-up sputum samples obtained from patients who grew the same species at baseline and showed the same molecular profile in both samples analysed by PFGE were considered long-term persistent strains, whereas PPMs cultured at follow-up which were not present in the baseline sample were considered as strains acquired during follow-up (fig. 1).

Inflammatory markers

The remaining sputum was centrifuged and the concentrations of several cytokines (tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8, IL-10 and IL-12) were measured in the supernatant using a cytokine bead array (BD Biosciences, San Diego, CA, USA). This assay system consists of a mixture of uniform bead types that contain different fluorescence intensities of a red-emitting dye. A capture antibody against each cytokine is covalently coupled to a type of bead, and cytokines bound to these antibodies are detected by use of phycoerythrin-labelled antibodies. The fluorescence intensity measured with phycoerythrin is proportional to the cytokine concentration in the sample and is quantified for every cytokine from a calibration curve. The detection limits of these assays were 3.7 pg·mL^{-1} for TNF-α, 7.2 pg·mL^{-1} for IL-1β, 2.5 pg·mL^{-1} for IL-6, 3.6 pg·mL^{-1} for IL-8, 3.3 pg·mL^{-1} for IL-10 and 1.8 pg·mL^{-1} for IL-12.

Statistical analysis

All data were added to a database and analysed using the SPSS statistical software package version 15 (Chicago, IL, USA). Results are expressed as absolute and relative frequencies for

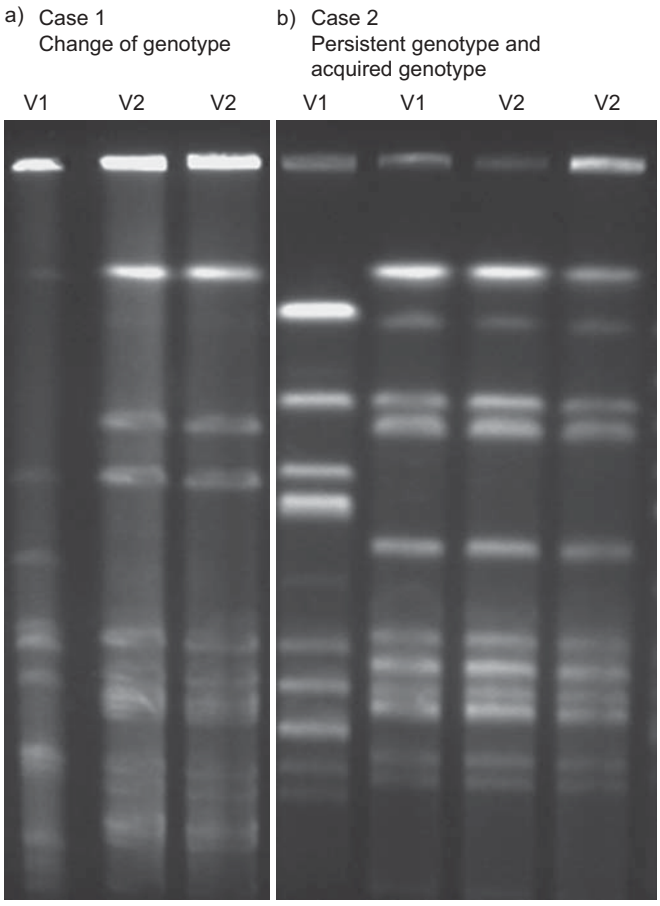

FIGURE 1. Pulse-field electrophoresis of samples growing *Haemophilus influenzae* in baseline and follow-up samples. a) Persistent *H. influenzae* strain, and b) appearance of a strain newly acquired during follow-up.

categoric variables and as means±SD, or median (interquartile range (IQR)) for continuous variables, as appropriate depending on the distribution of data. A value of half the lower detection limit was used for all measures of continuous variables showing a result below that value.

Descriptive statistics were compiled for sociodemographic characteristics and all the examinations were performed at baseline and at the end of follow-up. Sputum characteristics recorded at both visits were microbial profile and load, neutrophilia according to Murray-Washington criteria (⩾3), and concentration of cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12). Additional follow-up measurements were length of time in months between visits, appearance and frequency of exacerbations (calculated as number of exacerbations in the period/number of months of follow-up × 12); and change in the post-bronchodilator FEV1 in mL during the follow-up period (calculated as follow-up FEV1 - previous FEV1/number of months of follow-up × 12).

Analysis of the relationships between microbial typology and bronchial inflammation, measured as sputum neutrophilia and cytokine concentration, were performed at the end of follow-up (Chi-squared, Fisher's exact and Mann–Whitney U-tests). In this analysis, PPMs cultured from follow-up sputum samples and showing the same molecular profile as PPMs recovered at baseline were considered as long-term persistent strains, whereas PPMs cultured at follow-up but not present at baseline were considered as acquired. First, correlations between microbial load and bronchial inflammation parameters (Mann–Whitney U-test and the Spearman's correlation coefficient) were calculated. Next, the differences between colonised patients who harboured long-term persistent PPMs and microorganisms acquired during follow-up were analysed, through the assessment of the relationships between long-term microbial persistence and microbial profile, load, sputum neutrophilia and concentration of cytokines at the follow-up visit. Finally, the impact of bronchial inflammation on the change in post-bronchodilator FEV1 during the follow-up period was assessed using logistic regression modelling, considering an FEV1 decline greater than the median decline as the outcome, and age, current smoking, use of inhaled corticosteroids and appearance of an exacerbation during follow-up as covariates. The results of these analyses were expressed as crude and adjusted OR with 95% CI. Multivariate models were adjusted for smoking and other covariates showing an association with the outcome variable in the univariate models ($p<0.20$). All statistical tests were two sided, and a p-value of ⩾0.05 less was reported as statistically significant.

TABLE 1 Sociodemographic characteristics

Patients n	40
Age yrs	66.5±8.1
Males	39 (97.5)
Ever-smokers	40 (100.0)
Current	8 (20.0)
Former	32 (80.0)
Chronic bronchitis	28 (70.0)
Exacerbations previous yr	0 (0–2)
Use of inhaled steroids	29 (72.5)
Post-bronchodilator	
FEV1 %	57.9±19.1
FVC %	89.4±23.1
FEV1/FVC %	50.4±10.2
***P*a,O2 mmHg**	76.8 (10.9)
***P*a,CO2 mmHg**	40.5 (3.5)

Data are presented as mean±SD, n (%), or median (interquartile range), unless otherwise stated. FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; P_{a,O_2}: arterial partial pressure of oxygen; P_{a,CO_2}: arterial partial pressure of carbon dioxide.

RESULTS

40 consecutive, clinically stable COPD outpatients fulfilled the inclusion criteria and were enrolled. Participating patients were mostly male (39 (97.5%)), had a mean age±SD of 66.5±8.1 yrs and showed moderate lung function impairment (post-bronchodilator FEV1 57.9% of predicted (SD 19.1%)) (table 1). In these patients, 79 baseline visits and consecutive follow-up examinations were performed after 8.0±3.2 months. Sputum was obtained with isotonic saline in 23 baseline and 34 follow-up visits, and was induced with hypertonic saline in the remaining examinations. PPMs were recovered from nearly three-quarters of these sputum samples, both at baseline and at

TABLE 2 Characteristics of sputum sample (n=79)

	Sputum		
	Baseline	Follow-up	p-value
PPMs	58 (73.4)	56 (70.9)	>0.25
Sputum neutrophilia#	53 (67.1)	40 (50.6)	>0.25
PPMs and neutrophilia#	45 (57.0)	35 (44.3)	0.15
Polymicrobial	10 (12.7)	9 (11.4)	>0.25
Microbial typing			
H. influenzae	28 (35.4)	25 (31.6)	>0.25
Load cfu·mL^{-1} × 10^3	100 (20–10000)	700 (10–4500)	>0.25
P. aeruginosa or enterobacteria	18 (22.8)	22 (27.8)	>0.25
Load cfu·mL^{-1} × 10^3	2 (1–10)	1 (1–6)	>0.25
H. parainfluenzae	16 (20.2)	16 (20.2)	>0.25
Load cfu·mL^{-1} × 10^3	100 (10–115)	55 (6–165)	>0.25
M. catarrhalis	4 (5.1)	2 (2.5)	>0.25
TNF-α pg·mL^{-1}	9 (5–29)	18 (4–41)	>0.25
IL-1β pg·mL^{-1}	664 (173–1619)	837 (179–2493)	>0.25
IL-6 pg·mL^{-1}	692 (182–2694)	494 (120–1834)	>0.25
IL-8 ng·mL^{-1}	31 (8–44)	40 (23–65)	0.02
IL-10 pg·mL^{-1}	6 (4–13)	10 (3–27)	0.16
IL-12 pg·mL^{-1}	7 (1–17)	7 (1–23)	>0.25

Data are present as n (%) and median (interquartile range). PPM: potentially pathogenic microorganisms; *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *H. parainfluenzae*: *Hemophilus parainfluenzae*; *M. catarrhalis*: *Moraxella catarrhalis*; cfu: colony-forming unit; TNF: tumour necrosis factor; IL: interleukin. #: Murray-Washington score ⩾3.

TABLE 3 Evolution during follow-up (n=79)

Months follow-up	8.0±3.2
⩾1 exacerbation during follow-up	21 (26.6)
Exacerbations in follow-up per yr	0 (0–1)
Change FEV1 post-bronchodilation mL·yr^{-1}	-72 (-270–120)
Former smokers#	-50 (-390–126)
Current smokers#	-76 (-240–90)
Sputum cultures baseline and follow-up	
PPMs in both	42 (53.2)
Same PPM strain in both	12 (15.2)
PPMs and neutrophilia¶ in both	16 (20.3)
Same PPM strain and neutrophilia¶ in both	12 (15.2)

Data are presented as mean±SD, n (%) or median (interquartile range). FEV1: forced expiratory volume in 1 s; PPM: potentially pathogenic microorganisms. #: difference statistically nonsignificant (p>0.25, Mann–Whitney U-test). ¶: Murray-Washington score ⩾3.

the follow-up visits, and in half of them this recovery was associated with a neutrophilic response. *H. influenzae* was the PPM recovered most often (in 28 and 25 cases at baseline and follow-up visits, respectively), and samples growing that microorganism also had the highest microbial loads. Low loads of *P. aeruginosa*, enterobacteria and *H. parainfluenzae*, however, were also often cultivated from the sputum samples (table 2).

59 samples showed bronchial colonisation at the follow-up examination, preceded by positive sputum cultures for PPMs in the baseline sputum sample in 42 (53.2%) cases. These successive positive cultures, however, were in most cases due to new PPMs acquired during follow-up (30 (38.0%) cases). Long-term persistence of the same strain was the cause in only 12 (15.2%) cases, a situation that was mainly attributable to colonisation by *P. aeruginosa* or enterobacteria (table 3). No statistically significant differences in the measured bronchial inflammation parameters were found between sputum samples with long-term persistent and acquired colonisation (table 4).

The identification of bronchial colonisation by *H. influenzae* in the follow-up sputum was associated with a bronchial inflammatory response identifiable through a higher prevalence of sputum neutrophilia in samples positive for this PPM (68.0 *versus* 42.6%; p=0.04, Chi-squared test), and higher concentrations of IL-1β (median 1,636 (IQR 597–7,736) *versus* 601 (153–1,320) pg·mL^{-1}; p=0.005, Mann–Whitney U-test) and IL-12 (14 (7–29) *versus* 2 (1–17) pg·mL^{-1}; p=0.01, Mann–Whitney U-test) (fig. 2; table 5). This inflammatory response was not observed in samples colonised by *P. aeruginosa*/enterobacteria or *H. parainfluenzae* (table 5). The recovered load of *H. influenzae* was also positively correlated with the sputum concentrations of IL-1β in patients with sputum cultures positive for this PPM (ρ=0.64, p=0.001, Spearman's correlation coefficient) (fig. 3). In samples colonised by *P. aeruginosa* or enterobacteria, a neutrophilic inflammatory response was also observed (p=0.05, Chi-squared test) (table 5). This neutrophilic inflammatory response, mainly found in patients colonised by *H. influenzae*, *P. aeruginosa* or enterobacteria (27 out of 40 neutrophilic samples, 67.5%), showed a statistically significant relationship with FEV1 declines over the median decline during follow-up in our sample of moderate COPD patients (OR 2.67, 95% CI 1.07–6.62; p=0.03). That relationship was not modified after adjustment for covariates and was not found for IL-1β or IL-12, nor were statistically significant relationships found between inhaled corticosteroid use or appearance of an exacerbation and follow-up inflammatory markers in sputum (data not shown).

DISCUSSION

In the present study, focusing on the effects of bronchial colonisation on inflammation and lung function in moderate COPD, we found that PPMs were recovered from bronchial secretions in nearly three-quarters of the patients. The recovery of *H. influenzae*, *P. aeruginosa* and enterobacteria from bronchial secretions was closely related to a neutrophilic response, suggesting that the cellular inflammatory effects of bronchial colonisation on moderate COPD depend mainly on the presence of these PPMs. *H. influenzae* colonisation was additionally associated with higher sputum concentrations of such inflammation markers as IL-1β and IL-12, a relationship that was shown to be load-mediated for IL-1β. Bronchial colonisation by *H. parainfluenzae*, however, was not associated with an inflammatory response, a finding suggesting that the effect of this microorganism on the bronchial mucosa must be considered as marginal. The repetition of the sputum cultures was recurrently positive for PPMs in more than half of the

TABLE 4 Microbial load and inflammatory markers in follow-up samples showing colonisation according to persistence of potentially pathogenic microorganisms

	PPM strain		
	Persistent	Acquired	p-value
Samples n	12	44	
H. influenzae	5 (41.7)	20 (42.5)	>0.25
Load[#] cfu·mL^{-1} × 10^3	3000 (50–10000)	550 (10–2750)	0.23
***P. aeruginosa* or enterobacteria**	9 (75.0)	13 (27.7)	0.006
Load[#] cfu·mL^{-1} × 10^3	1 (1–10)	1 (1–3)	>0.25
H. parainfluenzae	2 (16.7)	14 (29.8)	>0.25
Load[#] cfu·mL^{-1} × 10^3	10, 60[¶]	55 (6–165)	>0.25
Inflammatory markers			
Sputum neutrophilia[+]	8 (66.7)	27 (61.4)	>0.25
TNF-α pg·mL^{-1}	21 (9–56)	19 (3–48)	>0.25
IL-1β pg·mL^{-1}	1404 (662–2790)	1095 (188–5323)	>0.25
IL-6 pg·mL^{-1}	664 (156–2335)	557 (151–2532)	>0.25
IL-8 ng·mL^{-1}	42 (15–55)	41 (24–92)	>0.25
IL-10 pg·ml^{-1}	14 (6–31)	10 (3–29)	>0.25
IL-12 pg·mL^{-1}	16 (9–26)	7 (1–21)	0.08

Data are presented as n (%) or median (interquartile range), unless otherwise stated. PPM: potentially pathogenic microorganisms; *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *H. parainfluenzae*: *Hemophilus parainfluenzae*; cfu: colony-forming units; TNF: tumour necrosis factor; IL: interleukin. [#]: considering only samples with cultures positive for the potentially pathogenic microorganism; [¶]: absolute values for the only two individual observations; [+]: Murray–Washington score ⩾3. Comparisons were performed using a Chi-squared, Fisher's exact or Mann–Whitney U-test as required.

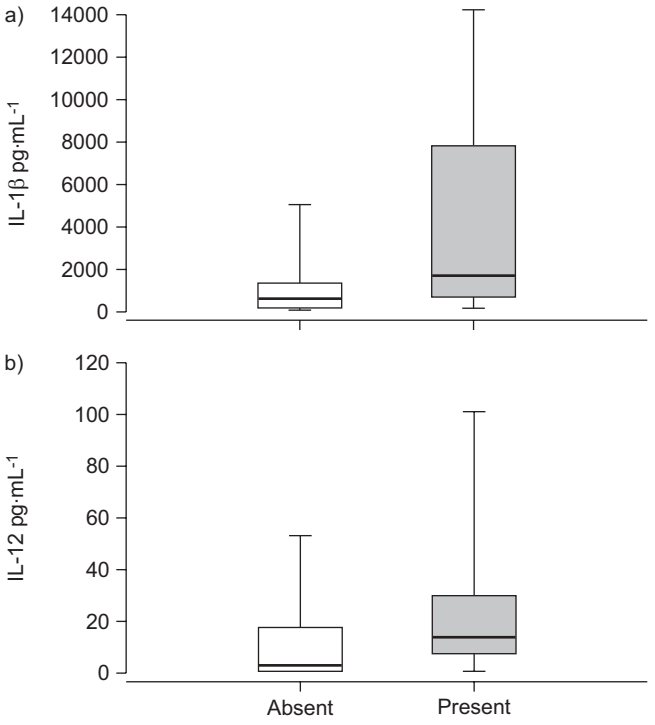

FIGURE 2. a) Interleukin (IL)-1β and b) IL-12 inflammatory markers in sputum according to the presence of bronchial colonisation by *Haemophilus influenzae*. Boxes represent median (horizontal dividing line) and interquartile range; whiskers represent SD.

patients, in most cases due to colonisation during follow-up by a newly acquired PPM; thus, long-term persistence of the same strain was observed in fewer than a fifth of the cases, all, however, with an associated neutrophilic response. The finding of sputum neutrophilia during stability was associated with a significant lung function decline in these moderate COPD patients, a relationship that was independent of current smoking.

Previous studies of bronchial colonisation in stable COPD patients, in which the protected specimen brush was used to collect microbiology samples, have demonstrated cultures positive for PPMs in one-third of the patients, with *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* the PPMs most often recovered [1]. When sputum has been used for the identification of bronchial colonisation in COPD, the cultures positive for PPMs have been found in one-fifth [5] to three-quarters [4] of the samples. In patients unable to spontaneously produce appropriate sputum samples during their stable periods, the use of sputum induction has facilitated the study of bronchial colonisation by allowing most stable COPD patients to be sampled [6, 27]. Study of the cytological characteristics of sputum in this context has shown cultures positive for PPMs with neutrophilia in half of the studied cases [3, 28], a rate similar to the prevalence found in the present study, in which *H. influenzae* was the most frequently isolated PPM.

Bronchial colonisation by *H. influenzae* was significantly related to sputum neutrophilia and higher sputum concentrations of the inflammatory mediators IL-1β and IL-12 in our sample of stable COPD patients with moderate lung function impairment. These observations suggest that colonisation by these PPMs is already able to induce an inflammatory response in the bronchial mucosa of such patients. This inflammatory response was seen to be species-specific because in our series, a similar effect was not found in COPD patients colonised by *H. parainfluenzae*, a PPM that occasionally causes COPD exacerbation and pneumonia [29–31], but has been shown to exhibit low mucosal adherence and a minimal potential to cause bronchial inflammation [32, 33]. Our choice of *H. influenzae* load as a main study variable was justified by its close relationship to the inflammatory response of the bronchial mucosa. Most PPMs recovered from participating COPD patients grew low microbial loads ($\leqslant 10^5$ cfu·mL^{-1}), with high microbial loads being found almost exclusively when *H. influenzae* was the colonising PPM in our study. High-load *H. influenzae* colonisation was significantly associated with neutrophilic sputum samples (Murray-Washington ⩾3) and higher levels of IL-1β in sputum. When dose–response relationships between PPM load and bronchial inflammatory mediators in sputum have been assessed in previous studies not focusing on *H. influenzae*, they have supported a causal role for bronchial colonisation in the pathogenesis of bronchial

TABLE 5 Inflammatory markers at follow-up according to sputum microbial typing (n=79)

	Colonisation by:								
	H. influenzae			*P. aeruginosa* or enterobacteria			*H. parainfluenzae*		
	Absent	Present	p-value#	Absent	Present	p-value#	Absent	Present	p-value#
Samples n	54	25		57	22		63	16	
Load cfu·mL^{-1} × 10^3		700 (10–4000)			1 (1–5)			5 (8–115)	
Sputum neutrophilia	23 (42.6)	17 (68.0)	0.04	25 (43.9)	15 (68.2)	0.05	30 (47.6)	10 (62.5)	>0.25
TNF-α pg·mL^{-1}	12 (4–33)	28 (4–56)	0.16	19 (4–43)	10 (5–46)	>0.25	19 (5–48)	14 (2–30)	>0.25
IL-1β pg·mL^{-1}	601 (153–1320)	1636 (597–7736)	0.005	601 (159–1809)	1126 (628–3630)	0.10	1037 (268–3956)	276 (113–830)	0.01
IL-6 pg·mL^{-1}	521 (67–1576)	463 (148–3539)	>0.25	468 (119–2326)	563 (186–1580)	>0.25	465 (126–1668)	647 (116–2421)	>0.25
IL-8 ng·mL^{-1}	38 (22–51)	41 (26–81)	0.24	37 (22–65)	41 (25–67)	>0.25	40 (25–65)	34 (16–61)	>0.25
IL-10 pg·mL^{-1}	9 (2–25)	14 (6–35)	0.09	12 (4–28)	6 (2–23)	>0.25	10 (4–26)	9 (2–29)	>0.25
IL-12 pg·mL^{-1}	2 (1–17)	14 (7–29)	0.01	7 (1–20)	9 (1–28)	>0.25	7 (1–26)	1 (1–10)	0.11

Data are presented median (interquartile range) or n (%), unless otherwise stated. *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *H. parainfluenzae*: *Hemophilus parainfluenzae*; cfu: colony-forming units; TNF: tumour necrosis factor; IL: interleukin. #: Comparisons were performed using a Chi-squared test, a Fisher's exact test or a Mann–Whitney U-test as required.

inflammation in COPD [4, 34, 35], and loads >10^5 cfu·mL^{-1} have usually been associated with neutrophilic inflammation [3]. Most series of patients with advanced disease, however, have shown positive correlations between microbial load and IL-8, myeloperoxidase, neutrophil elastase and leukotriene B_4 [3, 4, 28]. Together, all of these findings point to a significant impact of bronchial colonisation load on the mucosal inflammatory response, which, according to our results, may already be found, though with a different pattern, in patients with moderate disease when *H. influenzae* is the colonising bacteria. IL-1β has been related to bronchial colonisation in animal models [36], and our observation of high concentrations of this inflammatory mediator and IL-12, a cytokine related to the mucosal response against bacteria [37], in moderate COPD patients with *H. influenzae* colonisation supports the hypothesis of differences in the inflammatory response to bronchial colonisation according to the severity of lung function impairment. Our finding of a different pattern may have clinical implications and requires further assessment.

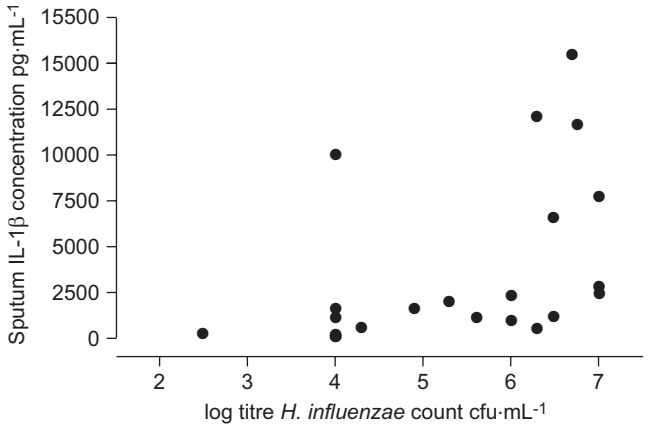

FIGURE 3. Scatterplot of total bacterial count (colony-forming units (cfu)·mL^{-1}) and sputum interleukin (IL)-1β (Spearman's correlation coefficient ρ=0.64, p=0.001) in patients colonised by *Haemophilus influenzae* at the follow-up examination (n=25). Bacterial count data have been logarithmically transformed.

We have found that samples showing bronchial colonisation were preceded by positive sputum cultures for PPMs in more than half the cases, and we have used molecular typing techniques to investigate whether recovered PPMs corresponded to newly acquired or long-term persistent strains. According to this analysis, bronchial colonisation was mostly attributable to new PPMs rather than to persistent ones. Persistent colonisation was observed in fewer than a fifth of the samples that were positive on follow-up and was most often attributable to long-term colonisation by *P. aeruginosa* or enterobacteria. Two previous studies reported that microbial persistence accounted for about a half of the cases of bronchial colonisation in stable COPD patients [3, 5], a prevalence higher than we have found. These studies, however, mostly investigated patients with severe COPD, and only one of them used molecular typing techniques to rule out colonisation by a new strain of the same PPM [5]. Our observations suggest that, in spite of a high prevalence of bronchial colonisation in moderate COPD patients, long-term microbial persistence is less often the cause, probably due to a higher turnover of colonising strains in less advanced disease.

Our findings in moderate COPD patients colonised by *H. influenzae*, *P. aeruginosa* and enterobacteria confirm the close association between colonisation by these PPMs and neutrophilia reported in other studies [34]. This association supports

an early relationship between colonisation and the appearance of cellular inflammatory mediators in bronchial secretions, years before the disease causes severe impairment and at a stage when these relationships could be more easily identifiable [38, 39]. The importance of the neutrophilic inflammatory response associated with bronchial colonisation is emphasised by our finding of a statistically significant relationship between the appearance of this inflammatory response and lung function impairment during follow-up. A similar functional impairment associated with bronchial colonisation has been documented by WILKINSON *et al.* [3], but was not found by HILL *et al.* [4]. In our study, the decline in FEV1 in COPD patients with sputum neutrophilia in some cases approached the magnitude previously seen in exacerbated COPD patients with more severe disease [40]. This finding supports the hypothesis that bronchial colonisation may have a subclinical impact in moderate COPD patients, who may be less prone to report their daily symptoms as an exacerbation [41]. Such patients may have functional impairment equivalent to the FEV1 decline that is associated with exacerbation in patients with advanced disease, who suffer easily from dyspnoea with even slight impairments in their lung function.

We have not performed genomic analysis of the sputum samples that do not grow PPMs, and we cannot rule out underdiagnosis of microbial persistence due to bronchial colonisation at loads below the detection limit of the sputum culture. This situation must be considered as unusual, however, because when this approach has been used undiagnosed, bronchial colonisation has only been identified in one-tenth of the culture-negative sputum samples [42]. The lack of virological data is also a limitation of our study, but respiratory viruses are seldom recovered from bronchial secretions in stable COPD patients, who only give positive samples in one-tenth of the cases [43, 44]. The lowest prevalence of viral recovery has been found in infrequent exacerbators [44], such as those in our study.

In summary, we found that the bronchial tree of clinically stable outpatients with moderate COPD and without previous admissions due to respiratory diseases is often colonised by PPMs, but high sputum loads are only reached by *H. influenzae*. The presence of this PPM is associated with an inflammatory response of the bronchial mucosa, characterised by neutrophilic inflammation and high levels of IL-1β and IL-12, findings that are not characteristic of patients colonised by *H. parainfluenzae*. Colonising *P. aeruginosa* and enterobacteria are more often persistent and also associated with sputum neutrophilia. These observations suggest that the effect of bronchial colonisation on COPD patients is mainly mediated by *H. influenzae*, *P. aeruginosa* and enterobacteria, PPMs that are able to cause an identifiable inflammatory response in moderately ill COPD patients.

SUPPORT STATEMENT

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STATEMENT OF INTEREST

None declared.

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M.E. Kerans (Translation Office, Barcelona, Spain) reviewed the English usage in the manuscript.

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2. **Estudi 2:** *Effect of bronchial colonisation on airway and systemic inflammation in stable COPD*

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Factor d'impacte 2012: 2.25

Resum

La finalitat d'aquet estudi és determinar la relació entre la colonització bronquial en els pacients amb MPOC en fase d'estabilitat clínica i la inflamació local i sistèmica. Es van incloure els 133 pacients procedents de la cohort PAC-EPOC dels quals es disposava de caracterització microbiològica, citològica i inflamatòria de l'esput, després d'un primer ingrés hospitalari per exacerbació de la MPOC . En fase d'estabilitat clínica, tres mesos després de l'ingrés es van recollir mostres d'esput per anàlisi microbiològic i detecció d'interleuquines a l'esput (IL-1β, IL-6 i IL-8), i mostres de sang per determinació de proteina-C-reactiva.

Es va trobar colonització bronquial en el 29% dels pacients. Aquesta colonització bronquial estava significativament associada a altes concentracions d'interleuquines a l'esput en comparació amb les mostres que no estaven colonitzades (IL-1β (mediana [percentil 25-75] 462 [121-993] *versus* 154 [41-477] pg/ml, p=0.002), IL-6 (147 [71-424] vs. 109 [50-197] pg/ml, p=0.047) i IL-8 (15 [9-19] *versus* 8 [3-15] ($\times 10^3$) pg/ml, p=0.002). També es van trobar altes concentracions de proteina-C-reactiva en sèrum en aquells pacients colonitzats respecte dels qui no ho estaven (6.5 [2.5 -8.5] *versus* 3.5 [1.7-5.4] mg/l,

p=0.016). La colonització bronquial per *Haemophilus influenzae* estava associada, així, a més inflamació pulmonar i sistèmica, i, a més, a pitjors puntuacions en els dominis d'impacte i activitat del qüestionari de qualitat de vida de St. George.

Resultats

Microbiologia i qualitat de l'esput

Un total de 155 pacients procedents de la cohort PAC-EPOC disposaven d'una caracterització microbiològica, citològica e inflamatòria completa de l'esput. D''aquests pacients, 133 (el 85.5%) van produir una mostra d'esput representativa de l'arbre traqueobronquial amb una baixa proporció de cèl·lules escatoses i es van incloure per l'estudi. Els pacients tenien una mitjana d'edat de 70 (DE 9) anys, una alteració de la funció pulmonar de grau moderat (mitjana de VEMS post broncodilatador de 52% del predit [DE 16]). Es van cultivar MPPs en 39 dels pacients (29%). La càrrega bacteriana era elevada en la majoria dels casos amb una mediana de 10^6 ufc/ml. Es van aïllar un total de 47 patògens, essent *Haemophilus influenzae* (n=22), *Pseudomonas aeruginosa* (n=8), *Moraxella catarrhalis* (n=6) i *Streptococcus pneumoniae* (n=5) els més freqüentment cultivats.

Colonització bronquial i inflamació pulmonar i sistèmica

Els pacients no colonitzats (n=94) i els colonitzats (n=39) no van mostrar diferències en variables sociodemogràfiques, clíniques o funcionals, excepte per una major exposició acumulada al tabac en els pacients colonitzats.
Es va observar una resposta bronquial neutrofílica similar en ambdós grups, però el grup dels pacients colonitzats va mostrar nivells més elevats de IL-1β (p=0.002, test de Kruskall-Wallis), IL-6 (p=0.047) i IL-8 (p=0.002). La colonització bronquial també es va associar amb discreta eosinofília a l'esput (p=0.015). Els nivells dels marcadors d'inflamació a l'esput no van mostrar associació amb la teràpia amb esteroides inhalats.

La presència de patògens a l'esput es va relacionar amb inflamació sistèmica mesurada per un increment de proteina-C-reactiva al sèrum (mediana 6.5 [2.5-

8.5] a colonitzats *versus* 3.5 [1.7-5.4] mg/l a no colonitzats, p=0.016). La proporció de pacients amb nivells plasmàtics de proteina-C-reactiva per sobre de 7.06 mg/l va ser significativament més gran al grup de pacients colonitzats (36% *versus* 18%, p=0.030, test de chi-quadrat). No es van trobar diferències estadísticament significatives en els nivells de fibrinogen i interleuquines plasmàtiques entre ambdós grups de pacients. La relació trobada entre els nivells elevats de proteina-C-reactiva i la colonització bronquial fou estadísticament significativa (OR 2.52, 95% IC 1.08-5.92, p=0.033) i es va mantenir després d'ajustar per edat i VEMS.

Colonització bronquial espècie específica

Haemophilus influenzae va ser el patogen més freqüentment aïllat. Es van comparar les característiques del grup de pacients colonitzats per *Haemophilus influenzae* (n=22) amb les del pacients no colonitzats i no es van trobar diferències significatives en quant a exacerbacions l'any previ o FEV1, però sí una exposició acumulada al tabac més alta en el grup de pacients colonitzats (103 [62-138] *versus* 58 [36-90] paquets-any; p=0.003, test de Kruskall-Wallis). Els pacients colonitzats per *Haemophilus influenzae* mostraren nivells bronquials més alts de IL-1β i IL-8 (p=0.001, test de Kruskall-Wallis). Els nivells de IL-8 estaven significativament correlacionats amb la càrrega bacteriana de *Haemophilus influenzae* a l'esput (coeficient de correlació r = 0.529, p=0.024, test de Spearman).

La colonització per *Haemophilus influenzae* s'associà a una pitjor qualitat de vida mesurada per el qüestionari de St.George, comparat amb els pacients no colonitzats. Aquesta associació era estadísticament significativa en els dominis d'activitat (58 [DE 26] *versus* 47 [DE 24], p=0.049, t de Student) i impacte (34 [DE 18] *versus* 25 [DE 18], p=0.041, t de Student). Aquests resultats només es van trobar en els colonitzats per *Haemophilus influenzae* i no en els colonitzats per altres patògens.

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ORIGINAL RESEARCH

Effect of Bronchial Colonisation on Airway and Systemic Inflammation in Stable COPD

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Abstract

The recovery of potentially pathogenic microorganisms (PPMs) from bronchial secretions is associated with a local inflammatory response in COPD patients. The objective of this study was to determine the relationships between bronchial colonisation and both bronchial and systemic inflammation in stable COPD. In COPD patients recruited on first admission for an exacerbation, bacterial sputum cultures, interleukin (IL)-1β, IL-6 and IL-8 levels, and blood C-reactive protein (CRP) were measured in stable condition. Bronchial colonisation was found in 39 of the 133 (29%) patients and was significantly related to higher sputum IL-1β (median [percentile 25–75]; 462 [121–993] vs. 154 [41–477] pg/ml, p = 0.002), IL-6 (147 [71–424] vs. 109 [50–197] pg/ml, p = 0.047) and IL-8 values (15 [9–19] vs. 8 [3–15] (×10³) pg/ml, p = 0.002). Patients with positive cultures also showed significantly elevated levels of serum CRP (6.5 [2.5–8.5] vs. 3.5 [1.7–5.4] mg/l, p = 0.016). Bronchial colonisation by *Haemophilus influenzae* was associated with higher levels of IL-1β and IL-8 and clinically significant worse scores on the activity and impact domains of the St. George's Respiratory Questionnaire. In conclusion, bronchial colonisation is associated with bronchial inflammation and high blood CRP levels in stable COPD patients, being *Haemophilus influenzae* related to a more severe inflammatory response and impairment in health-related quality of life.

Keywords: Haemophilus influenzae, IL-1β, IL-8, C-reactive protein, Health-related quality of life.

Introduction

In healthy subjects the bronchial tree and the pulmonary parenchyma are sterile, but potentially pathogenic microorganisms (PPMs) are often recovered from the bronchial secretions of patients with chronic obstructive pulmonary disease (COPD) during periods of clinical stability, with increased prevalence and higher loads appearing during episodes of exacerbation (1,2). Bronchial colonisation is identified in one third of the stable COPD patients (1), and has been related to an inflammatory response identifiable in bronchial secretions and blood, and quality of life impairment (3–5). The mechanisms behind this relationship are not yet well understood, however, and may be different depending on the colonising PPM (6–10).

Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease with subtypes that has been described in accordance with epidemiological

and clinical patterns (11–13). More recently, factor and cluster analysis (14–18) have been used for further assessment of the heterogeneity of the disease. The Phenotype and Course of Chronic Obstructive Pulmonary Disease (PAC-COPD) study has used these tools for the identification of COPD subtypes, in a cohort of 342 subjects hospitalised for the first time because of an exacerbation of the disease and examined three months after discharge, when clinically stable, validating prospectively three COPD subtypes against hospitalisations and mortality (19). Bronchial colonisation was similarly prevalent in the subtypes defined in the PAC-COPD Study, ranging between 26% and 36%, a finding that suggest that the relationships between bronchial colonization and COPD may be independent of the phenotype of the disease.

Starting with the hypothesis that bronchial colonisation in COPD is associated with bronchial and systemic inflammation, this study investigates the relationships between colonisation, bronchial and systemic inflammation in stable patients recruited at a first admission for disease exacerbation and enrolled in the PAC-COPD study (20), first focusing on the prevalence, typology and load of bronchial colonisation in COPD patients, and, second, investigating species-specific relationships between the colonising PPM and the observed inflammatory response, examining the associations between bronchial colonisation, inflammation and quality of life.

Methods

Design and participants

This cross-sectional analysis focusing on bronchial colonisation and inflammation in COPD was part of the population-based PAC-COPD study, that enrolled 342 COPD patients hospitalized for the first time for an exacerbation of their disease between January 2004 and March 2006 in 9 teaching hospitals in Spain, and further evaluated the patients in clinical stability. The recruitment process and the definitions of exacerbation, first admission, and COPD have been previously reported (20, 21). Current and former smokers included in the PAC-COPD study who expectorated samples with low squamous cell content and had complete information on sputum microbiology, cell counts and inflammatory markers at the baseline evaluation in clinical stability were selected for the present analysis, under the assumption that samples with 20% squamous cells or less were representative of tracheobronchial secretions (22). The research protocol was approved by the ethics committees of all the participating hospitals and written informed consent was obtained from all subjects.

Variables and measurements

Sociodemographic data were recorded at recruitment. Patients answered an epidemiologic questionnaire and performed all tests at least three months after hospital discharge and when clinically stable. Questions covered smoking habits, co-morbidity, respiratory symptoms, health-related quality of life with the St. George's Respiratory Questionnaire (SGRQ) and treatments. Patient functional characteristics included results of forced spirometry, reversibility testing, exercise capacity measured using the six minute walking test and body mass index (BMI).

Detailed information about the methods and the sources of the questionnaires and standardisation of the tests used in the PAC-COPD study has been previously published (20, 21).

Sputum induction

In patients unable to produce sputum spontaneously a sample was induced according to standard methods (23–25) and processed within 60 minutes of collection to guarantee cell viability (26). In brief, the patient was pre-treated with an inhaled ß-adrenergic agent 10 minutes before the nebulisation of increasing concentrations of hypertonic saline (0.9%, 3%, 4% and 5%), for 7 minutes each. Subjects were asked to blow their nose, rinse their mouth, and swallow water to minimize contamination from postnasal drip and saliva and the nebulisation procedure was interrupted when the sputum volume collected was 1 ml or more (27). After every induction the patient attempted to cough up sputum into a sterile plastic dish. The sputum sample obtained after the first induction was taken for microbiologic exam. The sputum samples obtained after a second induction were used for the analysis of inflammatory markers.

Microbiological exam

The sputum sample was weighed and processed with an equal volume of dithiothreitol (DTT) (Sputasol, Oxoid Ltd., Hants, UK), and cultured; the microbial load grown was then determined as described elsewhere (28). Microbiological processing included determination of microbial typology and load through serial dilutions and culture in selective media for potentially pathogenic microorganisms (PPMs), according to standard methods (29) with quantitative cultures expressed as colony-forming units (cfu) per millilitre.

In patients with polymicrobial cultures, the highest PPM load recovered was considered for the quantitative analysis. Cultures were considered positive for bronchial colonisation if they grew PPMs such as *Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Pseudomonas aeruginosa*, enterobacteria and/or *Staphylococcus aureus* according to previously defined criteria (30,31), at loads of at least 100 cfu/ml. *Haemophilus parainfluenzae* was not considered as a PPM in the present study. This bacteria has shown in cell cultures a low adherence to the bronchial mucosa (32), and in patients with chronic bronchitis has demonstrated poor ability to induce an airway inflammatory response (33).

Airway inflammatory markers and differential cell count

The sputum was separated from contaminating saliva by macroscopic examination. A Neubauer haemocytometer was used to determine cell viability using trypan blue exclusion. The remaining sputum was mixed with 4 times its weight of DTT solution, vortexed for 15 seconds, and then rocked for 15 minutes. A weight of phosphate-buffered saline solution equal to that of DTT was then added and the whole mixture was further vortexed for 15 seconds. The total inflammatory cell count, expressed as the absolute number of cells per gram of sputum, was calculated by subtracting squamous cells from the total cell count. Absolute and differential cell counts for neutrophils, lymphocytes and eosinophils were calculated by counting 400 non-squamous cells in Wright-stained slides. The remaining suspension was filtered through 48-µm nylon gauze and centrifuged at 750*g*.

The supernatant was decanted and stored at –80°C for later analyses. Cytokine concentrations (interleukin [IL] -1β, IL-6, IL-8) were measured in this sample using a cytokine bead array (BD Biosciences, San Diego CA, USA) following the manufacturer's instructions. The detection limits of these assays were 7.2 pg/ml for IL-1β, 2.5 pg/ml for IL-6 and 3.6 pg/ml for IL-8. All assays were performed in duplicate and, because intra-assay variation was always <10%, reported values correspond to the average of the 2 determinations.

Blood inflammatory markers

At the same visit for all participants, a peripheral venous blood sample (20 ml) was collected into tubes containing ethylene diamine triacetic acid after overnight fasting. The sample was centrifuged at 2000 rpm for 10 minutes, and the serum was stored at –80°C until analysis. Serum fibrinogen levels were measured by the Klauss method and C-reactive protein (CRP) levels by nephelometry. IL-6, IL-8 and tumour necrosis factor α (TNF-α) levels were determined with a high sensitivity enzyme-linked immunosorbent assay kit (Biosource, Camarillo, CA, USA). The lower limits of detection of these assays in serum samples were 0.16 mg/l, 0.104 pg/ml, 0.2 pg/ml and 0.09 pg/ml for CRP, IL-6, IL-8 and TNF-α respectively. All assays were performed in duplicate and reported values correspond to the average of the 2 determinations.

Statistical analysis

Data were analysed using the SPSS statistical software package version 15 (SPSS Inc., Chicago, IL, USA). Results for categorical variables are expressed as absolute and relative frequencies. Results for continuous variables are expressed as means (standard deviation [SD]) or medians (percentile 25–75 [P25-P75]) when the distribution was not normal.

First, sociodemographic, clinical and functional characteristics of no colonised and colonised patients were compared and the relationships between bronchial colonisation and inflammation were assessed in these patients using chi-square, Fisher exact, *t*-tests or Kruskal-Wallis tests, as required. Associations between sputum characteristics and systemic inflammation were examined through bivariate and multivariate analysis.

When CRP was considered as the outcome, the level was categorised using 7.06 mg/l as the cut-off, given that CRP levels over that value have been shown to be related to all-cause and cardiovascular mortality in COPD patients (34). Bivariate logistic regression was used to assess the associations between bronchial colonisation and high CRP levels, and covariate variables that showed a bivariate association ($p < 0.20$) were included in the multivariate analysis that assessed the relationship between bronchial colonisation and the outcome. Results were expressed as crude and adjusted odds ratios (OR) with 95% confidence intervals (95% CI). Finally, differential relationships between bronchial colonisation and patient characteristics, bronchial and systemic inflammation, and quality of life were assessed.

Levels of bronchial and systemic inflammatory markers and quality-of-life scores in patients colonized by *Haemophilus influenzae* were compared with results in non-colonised patients, repeating this comparison in patients colonised by other PPMs for this assessment. Correlations between bacterial loads and cytokine levels is sputum were also assessed in patients colonized by *Haemophilus influenzae*, using Spearman's rank correlation coefficients for the identification of dose-response relationships between bacterial load and bronchial inflammation. All statistical tests were two-sided, and a *p*-value of 0.05 or less was reported as statistically significant.

Results

Sputum quality and microbiology

One-hundred fifty-five participants in the PAC-COPD study had a complete microbiologic, cytological and inflammatory sputum characterisation available at baseline. One-hundred thirty-three of them (85.8%) produced sputum samples that showed a low proportion of squamous cells, and were considered as representative of tracheobronchial secretions. These samples also showed higher cell viability, counts and cytokine concentrations when compared with samples that contained more than 20% squamous cells on examination (data not shown, $p = 0.002$, Kruskal-Wallis test). In patients expectorating representative samples spontaneous (n = 76) and induced sputum (n = 57) gave similar results on cell counts and cytokine concentrations (data not shown, $p > 0.05$, Kruskal-Wallis test), and both samples were considered as equivalent for the purposes of this study.

Accordingly with these results, the 133 patients who had representative sputum samples were selected and included in the present analysis. They had a mean age of 70 (SD 9) years, a moderate lung function impairment

Table 1. Sociodemographic and sputum characteristics (microbiology, cytology and inflammatory markers) of PAC-COPD patients (n = 133)*

Patient characteristics	
Age (years), mean (SD)	70 (9)
Males, n (%)	124 (93)
Smoking pack-years, median (P25-P75)	67 (43–102)
Current smoker, n (%)	35 (27)
Exacerbation last year ≥1, n (%)	45 (34)
MMRC dyspnoea scale, mean (SD)	2.7 (1.3)
FEV1 post-BD% pred, mean (SD)	52 (16)
BMI (kg/m^2), mean (SD)	28 (5)
6MWD (m), median (P25-P75)	421 (369–457)
BODE index score, median (P25-P75)	3 (2–4)
SGRQ total score, mean (SD)	37 (18)
Inhaled corticosteroid treatment, n (%)	88 (66)
Sputum characteristics	
Cell viability (%), median (P25-P75)	80 (65–90)
Neutrophils/ml ×10^6, median (P25-P75)	6.4 (1.7–17.1)
Neutrophils%, median (P25-P75)	72 (48–84)
IL-1β (pg/ml), median (P25-P75)	209 (52–695)
IL-6 (pg/ml), median (P25-P75)	124 (54–259)
IL-8 (x103) (pg/ml), median (P25-P75)	11 (4–16)
Colonised, n (%)	39 (29)
Polymicrobial cultures, n (%)	8 (6)
Bacterial load ×106 (cfu/ml), median (P25-P75)†	8.1 (1.0–40.0)
Haemophilus influenzae	22 (17)
Pseudomonas aeruginosa	8 (6)
Moraxella catarrhalis	6 (4.5)
Streptococcus pneumoniae	5 (4)
Other PPMs‡	6 (4.5)

*Patients with representative sputum samples according to percentage of squamous cells≤20%.
†In patients with a positive culture for PPMs. Expressed as ×106 cfu/ml. PPM showing a higher load considered for patients with polymicrobial cultures.
‡*Staphylococcus aureus* and enterobacteria.
Definition of abbreviations: SD = standard deviation; MMRC = modified Medical Research Council; BD = bronchodilator; FEV_1 = forced expiratory volume in 1 second; BMI = body mass index; 6MWD = 6-minute walking distance; BODE = body-mass index (B), degree of airflow obstruction (O) and dyspnoea (D), and exercise capacity (E); PPMs = potential pathogenic microorganisms; cfu = colony forming units; IL = interleukin.
Some cases had missing values for certain variables: 3 patients cumulative smoking in pack-years; 8 6MWD; 8 BODE index; 8 bacterial load values.

(mean post-bronchodilator forced expiratory volume in 1 second [FEV_1] 52% [SD 16] of predicted), and a well-defined neutrophilic inflammatory pattern in sputum (median 72% [48%–84%]) (Table 1). PPMs were cultured from the sputum of 39 of these patients (29%). The bacterial load was high in most cases, with a median count over 10^6 colony forming units per millilitre. Forty-seven PPMs were recovered from these 39 patients, *Haemophilus influenzae* (n = 22), *Pseudomonas aeruginosa* (n = 8), *Moraxella catarrhalis* (n = 6) and *Streptococcus pneumoniae* (n = 5) being the bacteria most often cultivated (Table 1).

Bronchial colonisation and pulmonary and systemic inflammation

Clinical and sputum characteristics of no colonised (n = 94) and colonised (n = 39) COPD patients are compared in Table 2. Sociodemographic, clinical and functional differences between the two groups were not significant, except for higher cumulative smoking in colonised patients. A similar neutrophilic bronchial inflammatory response was observed in both no colonised and colonised patients, but higher values of IL-1β (p = 0.002, Kruskall-Wallis test), IL-6 (p = 0.047) and IL-8 (p = 0.002) were found in colonised. Bronchial colonisation was also significantly related to sputum eosinophilia in both absolute and relative counts (p = 0.015).

Inflammatory markers in sputum did not show significant associations with inhaled corticosteroid therapy in the studied patients. Eosinophil% in sputum samples from patients using inhaled corticosteroids (median [percentile 25–75] 1.2 [0–3.3] showed statistically non-significant differences when compared with the level of this inflammatory marker in patients not using this treatment (0.4 [0–3.9], p = 0.337, Kruskal-Wallis test). Similar results were obtained on the levels of IL-1β and IL-8 (data not shown, p = 0.567 and p = 0.530, respectively).

Bronchial colonisation was associated with systemic inflammation identifiable through an increase in blood CRP concentration (median 6.5 [2.5–8.5] in colonised vs. 3.5 [1.7–5.4] mg/l in non colonised patients, p = 0.016). The proportion of patients with plasma levels of CRP over 7.06 mg/l was also significantly higher in colonised patients (36% vs. 18%, p = 0.030, chi-square test). No significant differences, however, were detected in fibrinogen and plasma cytokine (IL-6, IL-8, and TNF-α) levels between no colonised and colonised patients.

The relationship found between bronchial colonisation and high CPR plasma levels was statistically significant (OR 2.52, 95% CI 1.08–5.92, p = 0.033) (Table 3), and maintained after the adjustment for age and FEV_1%. The risk of having a CRP concentration over 7.06 mg/l more than doubled in COPD patients who were colonised by PPMs in the multivariate model (adjusted OR 2.57, 95% CI 1.07–6.18).

Species-specific relationships of bronchial colonisation

H. influenzae was the most prevalent colonising PPM. When the characteristics of patients colonised by this PPM (n = 22) were compared with no colonised patients, post bronchodilator FEV_1% and frequency of exacerbation in the previous year were similar, even though patients colonised by *H. influenzae* had significantly more cumulative smoking (103 [62–138] vs. 58 [36–90] pack-years; p = 0.003, Kruskall-Wallis test). *H. influenzae*-colonised patients had significantly higher median cytokine values for both IL-1β and IL-8 (p = 0.001, Kruskal-Wallis test). The level of IL-8 were

significantly correlated with the load of *H. influenzae* in the sputum sample (correlation coefficient r = 0.529, p = 0.024, Spearman rank test); see Figure 1

Bronchial colonisation by *H. influenzae* was associated with a worse health-related quality of life when compared with no colonised patients (SGRQ total score 42 [SD 20] versus 35 [SD 17], p = 0.105, Student's *t*-test), a comparison that reached statistically significance in the activity (58 [SD 26] versus 47 [SD 24], p = 0.049, Student t test) and impact (34 [SD 18] versus 25 [SD 18], p = 0.041, Student's *t*-test) domains of the questionnaire. These results were not found when other PPMs are the colonisers of the lower airway (Table 4).

Discussion

We have investigated the characteristics of bronchial colonisation and its relationships with bronchial and systemic inflammation in a large, well-characterised sample of COPD patients in stable clinical condition about 3 months after recruitment during their first admission for an exacerbation. Sputum samples with a low proportion of squamous cells were recovered from most patients (85.8%). These samples were of high quality, showed high cell viability, and were considered representative of tracheobronchial secretions. Bronchial colonisation by PPMs was identified in 29% of these sputum samples, and was associated with a well-defined local inflammatory

Table 2. Patient characteristics, sputum characteristics and plasma inflammatory markers according to bronchial colonisation*, in PAC-COPD patients (n = 133)

	No. colonised n = 94	Colonised n = 39	*P*-Value
Patient characteristics			
Age (years), mean (SD)	69 (9)	70 (8)	0.669
Smoking pack-years, median (P25-P75)	58 (36–90)	80 (56–116)	0.007
Current smoker, n (%)	24 (27)	11 (28)	0.829
Chronic phlegm, n (%)	58 (62)	25 (64)	0.795
Exacerbation last year ≥1, n (%)	29 (31)	16 (41)	0.259
MMRC dyspnoea scale, mean (SD)	2.7 (1.4)	2.8 (1.3)	0.481
FEV1 post-BD% pred, mean (SD)	52 (17)	52 (14)	0.982
BMI (kg/m^2), mean (SD)	28 (5)	28 (4)	0.913
6MWD (m), median (P25-P75)	421 (374–461)	422 (367–452)	0.783
BODE index score, median (P25-P75)	3 (2–4)	3 (2–3)	0.806
SGRQ total score, mean (SD)	35 (18)	40 (20)	0.206
Inhaled corticosteroid treatment, n (%)	64 (68)	24 (62)	0.468
Sputum characteristics			
Total cells/ml ×10^6, median (P25-P75)	13 (4–23)	9 (4–22)	0.553
Neutrophils/ml ×10^6, median (P25-P75)	7 (2–18)	6 (2–13)	0.786
Eosinophils/ml ×10^6, median (P25-P75)	0.1 (0–0.3)	0.2 (0–0.7)	0.015
Neutrophils%, median (P25-P75)	71 (48–82)	75 (45–87)	0.524
Eosinophils%, median (P25-P75)	1 (0–3)	2 (0–5)	0.015
Eosinophils ≥3%, n (%)	22 (23)	16 (41)	0.041
IL-1β (pg/ml), median (P25-P75)	154 (41–477)	462 (121–993)	0.002
IL-6 (pg/ml), median (P25-P75)	109 (50–197)	145 (71–414)	0.047
IL-8 (x103) (pg/ml), median (P25-P75)	8 (3–15)	1315 (9–19)	0.002
Plasma inflammatory markers			
IL-6 (pg/ml), median (P25-P75)	1.1 (0.5–2.0)	1.1 (0.6–2.0)	0.985
IL-8 (pg/ml), median (P25-P75)	4.2 (3.3–5.5)	4.7 (3.1–7.2)	0.173
TNF-α (pg/ml), median (P25-P75)	0.2 (0–0.5)	0.3 (0–0.7)	0.589
CRP (mg/l), median (P25-P75)	3.5 (1.7–5.4)	6.5 (2.5–8.5)	0.016
CRP > 7.06 mg/l, n (%)	16 (18)	14 (36)	0.030
Fibrinogen (g/l), median (P25-P75)	3.9 (3.2–4.5)	4.0 (3.5–4.6)	0.204

*Bronchial colonisation defined as a sputum culture positive for *Haemophilus influenzae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus aureus* or enterobacteria.
Definition of abbreviations: IL = interleukin; TNF-α = tumour necrosis factor-α; CRP = C-reactive protein.
Some cases had missing values for certain variables: 3 patients cumulative smoking in pack-years; 8 6MWD; and 8 BODE index. Values were missing for plasma IL-6 in 7 cases, for plasma IL-8 in 6, for TNF-α in 5, for CRP in 6, and for fibrinogen in 5.

Table 3. Risk factors for high levels of plasma C-reactive protein (CRP) in PAC-COPD patients (n = 133)

	CRP ≤ 7.06 mg/l	CRP > 7.06 mg/l	Crude OR (95% CI)	*P*
n	97	30		
Age yrs, mean (SD)	69 (8)	71 (9)	1.03 (0.98–1.09)	=.190
Males, n (%)	91 (94)	28 (93)	1.08 (0.21–5.67)	=.924
Pack-year (over median), n (%)	52 (55)	17 (57)	1.06 (0.46–2.42)	=.897
Current smoker, n (%)	25 (27)	8 (27)	1.00 (0.40–2.54)	=.994
Chronic phlegm, n (%)	60 (62)	18 (60)	0.93 (0.40–2.14)	=.860
Exacerbation last year ≥1, n (%)	32 (33)	13 (43)	1.55 (0.67–3.59)	=.302
≥2 Co-morbidity†, n (%)	59 (61)	17 (57)	0.84 (0.37–1.93)	=.685
Cardiac co morbidity‡, n (%)	14 (14)	7 (23)	1.80 (0.65–4.99)	=.256
FEV_1 post-BD% pred, m (SD)	54 (17)	48 (11)	0.97 (0.94–1.00)	=.050
BMI (kg/m^2), m (SD)	28 (5)	28 (4)	0.99 (0.90–1.08)	=.770
SGRQ total score, mean (SD)	35 (18)	39 (18)	1.01 (0.99–1.04)	=.299
Bronchial colonisation*, n (%)	25 (26)	14 (47)	2.52 (1.08–5.89)	=.033

†From Charlson index ≥1 means one or more in addition to COPD.
‡Cardiac co-morbidity: Myocardial infarction and/or heart failure.
*Bronchial colonisation defined as a sputum culture positive for Haemophilus influenzae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus aureus or enterobacteria.
Definition of abbreviations: SD = standard deviation; FEV1 = forced expiratory volume in one second; BMI = body mass index.

response, as shown by higher levels of IL-1β, IL-6 and IL-8.

Colonised patients additionally showed a systemic inflammatory response, identifiable through higher concentrations of blood CRP. A species-specific relationship between bronchial colonisation by *H. influenzae* and IL-1β and IL-8 sputum levels was also detected, a bronchial inflammatory pattern that emerged to be related to worse health-related quality of life. Higher scores in the activity and impact domains of the St. George's Respiratory Questionnaire were observed in patients colonised by *H. influenzae*, suggesting that this PPM may have more far-reaching effects than others. Overall, these findings confirm the importance of the relationships

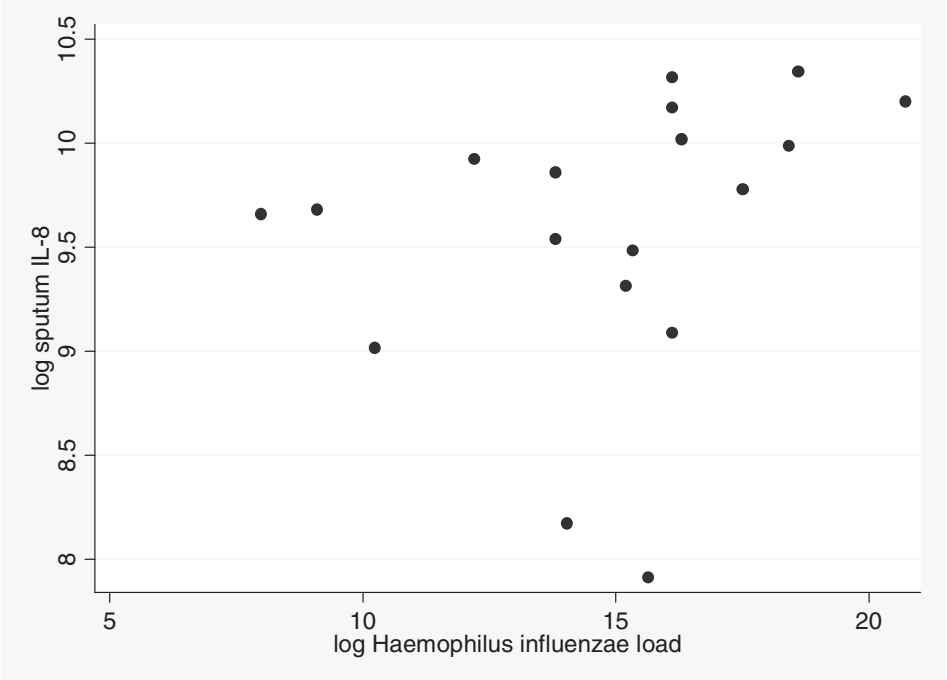

Figure 1. Scarplot of total bacterial count (colony forming units (cfu)/ml^{-1}) and sputum interleukin (IL)-8 (pg/ml^{-1}) (Spearman's correlation coefficient r = 0.529, $p = 0.024$) in patients colonised by *Haemophilus influenzae*.

Table 4. Species-specific relationships of bronchial colonisation (n = 133)

	No. colonised	*H. influenzae*	P value	Other PPMs[&]	*P*-value
No. of patients	94	22		17	
Bacterial load (x10^6) (cfu/ml), median (P25-P75)[†]	–	5.4 (1–12)	–	10 (1–100)	
Patient characteristics					
FEV_1 post-BD% pred, mean (SD)	52 (17)	51 (15)	0.647	55 (11)	0.558
Smoking pack-years, median (P25-P75)	58 (36–90)	103 (62–138)	0.003	68 (56–90)	0.322
Exacerbation last year ≥1, n (%)	29 (31)	7 (32)	0.930	9 (53)	0.077
SGRQ total score, mean (SD)	35 (17)	42 (20)	0.105	37 (20)	0.807
SGRQ activity score, mean (SD)	47 (24)	58 (26)	0.049	47 (24)	0.918
SGRQ impact score, mean (SD)	25 (18)	34 (18)	0.041	27 (21)	0.751
SGRQ symptoms score, mean (SD)	47 (19)	49 (14)	0.592	48 (22)	0.827
Sputum and plasma inflammatory markers					
Sputum IL-1β (pg/ml), median (P25-P75)	154 (41–477)	746 (121–1742)	0.001	279 (168–695)	0.202
Sputum IL-8 (×10^3) (pg/ml), median (P25-P75)	8 (3–15)	16 (11–22)	0.001	13 (6–16)	0.240
Plasma CRP (mg/l), median (P25-P75)	3.5 (1.7–5.4)	6.5 (2.5–7.7)	0.050	6.0 (2.7–14.0)	0.085

*Other potentially pathogenic microorganisms (PPMs) were *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Staphylococcus aureus* oand enterobacteria, in patients with negative cultures for *Haemophilus influenzae*.
[†]Expressed as ×10^6 cfu/ml. PPM showing a higher load considered for patients with polymicrobial cultures.
Patients colonised by *H.influenzae* were compared with no colonised patients. Patients colonised by other PPMs similarly compared with no colonised patients. Comparisons were performed using a chi-square, Fisher exact, *t* or Kruskal-Wallis tests, as required, using no colonised patients as the reference.
Definition of abbreviations: PPM = potentially pathogenic microorganisms; SGRQ = St. George's Respiratory Questionnaire.

between colonisation, bronchial and systemic inflammation, and identify species-specific mechanisms that could be targeted for management in COPD patients.

Median total cell counts in colonised and no colonised COPD patients were similar in our study (9 [4–22]/ml × 10^6 vs. 13 [4–23]/ml ×10^6), and associated with prevalence of sputum neutrophilia higher than 65%, a value considered as the upper limit of normality (24, 35), a finding also reported by Barnajee et al. (5). Neutrophilic inflammation has been related to colonisation in studies that have used bronchoalveolar lavage to sample peripheral lung secretions of stable COPD patients (6–8), a difference that may be related to specific characteristics of the cellular compartments targeted by bronchoalveolar lavage.

This last explanation is supported by studies that have found a higher proportion of neutrophils in sputum and bronchial aspirates recovered from COPD patients, in comparison with bronchoalveolar lavage fluid (36), suggesting that the effect of colonisation on the neutrophilic response in the bronchial mucosa is overwhelmed by the magnitude of inflammation associated with COPD. Colonised COPD patients enrolled in the present study showed a mild eosinophilic inflammatory response in sputum, with percentages of patients with sputum eosinophilia over 3% slightly higher in colonised than in no colonised patients, with values similar to figures reported for stable COPD (35, 37–41). Our findings suggest that the effect of colonisation on the cellular inflammatory response in bronchial secretions in stable COPD patients may be minimized by the magnitude of the local inflammation associated with the disease.

Increased levels of IL-1β and IL-8 in sputum were found to be associated with bronchial colonisation in our stable COPD patients with moderate disease. This finding supports the capability of PPMs to induce bronchial inflammation, as previously reported in severe patients (7, 8, 42). IL-8 is a well-known neutrophil chemo-attractant and activator (43), and the relationship between bronchial colonisation and the level of this cytokine in the sputum supernatant has been previously reported in both cell models (44) and COPD patients (45, 46), with a dose-response relationship to bacterial load in several studies (5, 10). IL-1 is a family of cytokines that comprises 11 proteins that have demonstrated a central role in a number of inflammatory diseases (47). IL-1 is a non-specific mediator (48) and elevated levels of IL-1β have also been reported as a response to the presence of PPMs in cell cultures and sputum samples from COPD patients (3).

COPD exacerbations are known to involve high levels of blood CRP (49, 50) and fibrinogen (51), and it is also widely accepted that COPD patients show a systemic inflammation pattern during their periods of stability. Gan et al. (4), in a systematic review, reported significantly raised levels of CRP in stable COPD patients when compared with healthy controls, and in these patients elevated CRP has been a predictor of COPD hospitalisation and death (52). Man et al. (34), studying the large cohort of COPD patients enrolled in the Lung Health Study, observed that patients attaining CRP values over 7.06 mg/l, are at risk for the appearance of cardiovascular events and death, when compared with COPD patients with low CRP concentrations.

In our study CRP levels found in no colonised patients (median 3.5 [1.7–5.4] mg/l) were similar to the values observed in other studies of stable COPD patients (50). However, a much higher level (6.5 [2.5–8.5] mg/l) was observed in colonised patients, over one third of whom reached values exceeding 7.06 mg/l. The association continued to be statistically significant after the adjustment for covariates (adjusted OR 2.57, 95% CI 1.07–6.18), FEV_1% being the only other measure also demonstrating a statistically significant inverse relationship with CRP elevation.

These findings support the hypothesis of a direct effect of bronchial colonisation on systemic inflammation. The elevation in blood CRP concentration observed when PPMs colonise the bronchial mucosa would be a risk factor that would remain undetected unless sputum cultures are performed during periods of stability.

Colonisation by *H. influenzae* was associated in our study with higher cumulative smoking, in spite of similar current smoking prevalence and lung function. This PPM was also associated with a clearly higher bronchial inflammatory response, as shown by much higher levels of IL-1β and IL-8, and a dose-response relationship was demonstrated between *Haemophilus influenzae* load and IL-8 levels in sputum. Thus, colonising *H. influenzae* may have species-specific effects identifiable through higher cytokine levels in bronchial secretions. In patients who had sputum cultures positive for this PPMs we observed an impaired health-related quality of life as measured by the activity and impact domains of St. George's Respiratory Questionnaire, a relationship that did not reach statistical significance when other PPMs are the colonisers. These associations were consistent with previous findings reporting significantly worse health status in colonised stable COPD patients with sputum cultures positive for *H. influenzae* (5).

The selection of patients used in the PAC-COPD study places limits on the extrapolation of the results, which cannot be applied to more severe patients because participants in this study had to have been hospitalised only once for a COPD exacerbation. This approach, however, determines that bronchial colonisation, the main variable considered in the present study, was not modified by recurrent admissions, which introduce in-hospital strains into the colonising flora. Thus, this apparent limitation in fact increases the strength of conclusions based on the observed relationships for COPD outpatients.

Additionally, we have not performed genomic analysis of the sputum samples not growing PPMs, and we cannot rule out under diagnosis of bronchial colonisation at loads below the detection limit of the sputum culture. Such colonisation should be considered as unusual, however, because when this approach has been used undiagnosed bronchial colonisation has only been identified in one tenth of the culture-negative sputum samples (6).

In conclusion, the present study confirms the relationship between bronchial colonisation and both local and systemic inflammation in stable COPD patients. Local inflammation is identifiable in colonised patients through high levels of IL-1β, IL-6, IL-8 and, to a lesser degree, through systemic inflammation indicated by raised blood CRP levels. The magnitude of systemic inflammation, however, is great enough to have significant effects on the course of disease. Moreover, a species-specific effect on bronchial inflammation patterns was observed, suggesting that the impact of colonisation by *H. influenzae* may be expected to exceed that of other PPMs. If these findings are confirmed by further research, there would be a theoretical basis for targeting bronchial colonisation with specific management approaches in COPD patients that would have a potential impact on prognosis.

Declaration of Interest

The authors report no conflicts of interest. The authors are responsible for the content and the writing of this paper.

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3. **Estudi 3:** *Colour of sputum is a marker for bacterial colonisation in chronic obstructive pulmonary disease*

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Respiratory Research 2010, 11:5 8

Factor d'impacte 2010: 2.859

Resum

La finalitat d'aquest estudi va ser identificar factors de risc per colonització bronquial en pacients amb MPOC en fase d'estabilitat clínica. Es van avaluar un total de 119 pacients dels quals 92.5% eren homes, amb una mitjana de edat de 68 anys i una mitjana de FEV1 de 46% del predit. Es va demostrat colonització bronquial a 58 pacients (48.7%). El grup de pacients colonitzats presentava més exposició acumulada al tabac, més exacerbacions i més ingressos hospitalaris l'any previ, major grau de dispnea i color més fosc a l'esput, en comparació amb el grup de pacients no colonitzats. Mes del 80% dels esputs de color groc fosc o verd presentaven MPPs. En contrast, només en el 5.9% del esputs de color blanc i en el 44.7% dels de color groc clar hi creixien patógens. L'anàlisi multivariant va mostrar un grau de dispnea elevat (OR=2.63, IC 95% 1.53-5.09, p=0.004) i color fosc a l'esput (OR 4.11, IC 95% 2.30-7.29, p<0.001) com a factors associats a la presència de colonització bacteriana a l'esput.

Resultats

Es van estudiar 119 pacients (92.5% homes), amb una mitjana (DE) d'edat de 68.1 (9.1) anys. Es va demostrar colonització bronquial en 58 (48.7%) pacients, per un sol bacteri en 50 casos i com a colonització múltiple en 8. *Haemophilus influenzae* i *Haemophilus parainfluenzae* van ser els patògens més freqüentment aïllats (72%). Es van trobar diferències significatives en consum de tabac, tos, dispnea, comorbiditats, exacerbacions i hospitalitzacions a l'any previ, i en el color de l'esput, entre els pacients amb i sense colonització bronquial.

En quant a la distribució de pacients en funció del color de l'esput, les mostres amb el color 1 en la escala utilitzada (blanc) eren predominantment estèrils, mentre que les mostres amb els color 3 al 5 (groc i verd) mostraren una prevalença de colonització superior al 80%. El color 2 (groc clar) no discriminava entre colonitzats i no colonitzats. Quan es van classificar els pacients d'acord amb la càrrega bacteriana, 36 pacients presentaven càrregues bacterianes elevades ($>10^5$ ufc/ml), mentre que els 22 restants tenien càrregues bacterianes baixes ($\leq 10^5$ ufc/ml). Les característiques dels pacients amb càrregues bacterianes altes (n=36) es van comparar amb el grup format per pacients no colonitzats (n=61) i els colonitzats amb càrregues bacterianes baixes (n=22) considerats junts (n=83). Les diferències estadísticament significatives en l'exposició al tabac acumulada, tos, grau de dispnea, hospitalitzacions l'any previ i color de l'esput, persistia al comparar els pacients amb càrregues bacterianes altes i la resta de pacients.

Pel que fa a l'anàlisi de marcadors d'inflamació a l'esput, es va poder obtenir suficient mostra en 61 casos. Les concentracions dels marcadors d'inflamació van mostrar una gran variabilitat inter-individual i no seguien una distribució normal. No es van trobar diferències significatives en les concentracions de cap dels marcadors d'inflamació a l'esput analitzats entre els pacients colonitzats i

els no colonitzats. Quan es va fer la mateixa comparació entres els pacients colonitzats amb càrregues bacterianes elevades i la resta, tots el marcadors d'inflamació, excepte la IL-6, presentaven major concentracions, encara que la diferència no arribava a la significació estadística.

Els resultats de l'anàlisi multivariant van ser molt similars quan s'identificaven els factors associats amb la presència de MPPs o quan es classificaven els pacients d'acord amb la càrrega bacteriana. En ambdós casos, només el grau de dispnea i el color de l'esput estaven significativament i independentment associats amb la presència de MPPs i càrregues bacterianes altes.

RESPIRATORY RESEARCH

RESEARCH Open Access

Colour of sputum is a marker for bacterial colonisation in chronic obstructive pulmonary disease

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Abstract

Background: Bacterial colonisation in chronic obstructive pulmonary disease (COPD) contributes to airway inflammation and modulates exacerbations. We assessed risk factors for bacterial colonisation in COPD.

Methods: Patients with stable COPD consecutively recruited over 1 year gave consent to provide a sputum sample for microbiologic analysis. Bronchial colonisation by potentially pathogenic microorganisms (PPMs) was defined as the isolation of PPMs at concentrations of $\geq 10^2$ colony-forming units (CFU)/mL on quantitative bacterial culture. Colonised patients were divided into high ($>10^5$ CFU/mL) or low ($<10^5$ CFU/mL) bacterial load.

Results: A total of 119 patients (92.5% men, mean age 68 years, mean forced expiratory volume in one second [FEV_1] [% predicted] 46.4%) were evaluated. Bacterial colonisation was demonstrated in 58 (48.7%) patients. Patients with and without bacterial colonisation showed significant differences in smoking history, cough, dyspnoea, COPD exacerbations and hospitalisations in the previous year, and sputum colour. Thirty-six patients (62% of those colonised) had a high bacterial load. More than 80% of the sputum samples with a dark yellow or greenish colour yielded PPMs in culture. In contrast, only 5.9% of white and 44.7% of light yellow sputum samples were positive ($P < 0.001$). Multivariate analysis showed an increased degree of dyspnoea (odds ratio [OR] = 2.63, 95% confidence interval [CI] 1.53-5.09, $P = 0.004$) and a darker sputum colour (OR = 4.11, 95% CI 2.30-7.29, $P < 0.001$) as factors associated with the presence of PPMs in sputum.

Conclusions: Almost half of our population of ambulatory moderate to very severe COPD patients were colonised with PPMs. Patients colonised present more severe dyspnoea, and a darker colour of sputum allows identification of individuals more likely to be colonised.

Background

Exacerbations are the main cost driver in chronic obstructive pulmonary disease (COPD), have a negative impact on the clinical course of the patients and are associated with increased mortality [1-3]. Around 70% of exacerbations are infectious in nature, either bacterial, viral or mixed [4-7]. It has been shown that airway bacterial load in the stable state contributes to airway inflammation and modulates the character and frequency of exacerbations [8,9]. There is also evidence that bronchial colonisation influences the decline in lung function over time [10]. Different studies in which respiratory samples were obtained by the protected specimen brush (PSB) technique have shown a high prevalence of bronchial colonisation in COPD patients [5,11,12]. However, the practice of bronchoscopy to assess bronchial colonisation in routine clinical practice is not feasible and data that support the use of sputum samples to identify patients colonised by potentially pathogenic microorganisms (PPMs) are required.

Consequently, a cross-sectional study was designed to assess the frequency of bronchial bacterial colonisation using sputum samples and to identify risk factors for colonisation in stable ambulatory patients with COPD. The

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clinical characteristics of patients colonised and non-colonised with PPMs were compared as were those of patients with low and high bacterial loads in sputum samples.

Methods

A cross-sectional study was carried out to assess clinical characteristics associated with bronchial colonisation in stable ambulatory COPD patients. These patients were visited at the outpatient respiratory clinics of two acute-care tertiary hospitals in Barcelona, Spain and were consecutively recruited over one year. After completing the collection of data for this study, patients with bronchial colonisation were included in a randomised trial of antibiotic treatment the results of which have been reported elsewhere [13]. The protocol was approved by the institutional review board and all patients gave written informed consent.

Study population

Eligible patients were adults over 40 years of age, smokers or ex-smokers of at least 10 pack-years, with stable COPD, defined as a post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ratio of <70%. A FEV_1 of <60% of the predicted value higher than 0.70 litres and a negative bronchodilator test (increase in FEV_1 <200 mL and <12% of baseline) was required for inclusion in the study as was a history of at least one documented exacerbation in the previous year. Clinical stability was defined by the attending physician on clinical grounds based on the absence of symptoms of exacerbation and use of any oral or systemic antibiotics or a course of oral corticosteroids in the 6 weeks prior to inclusion.

The exclusion criteria were the following: (1) previous diagnosis of bronchial asthma, bronchiectasis demonstrated by a chest X-ray or computed tomography (CT) scan, or other relevant pulmonary diseases apart from COPD; (2) chronic treatment with oral corticosteroids at any dose; (3) formal contraindication for sputum induction or impossibility to obtain a valid sputum sample for analysis; and (4) participation in another clinical study concurrently or within the previous 3 months.

Study procedures

At the time of inclusion in the study, the investigator verified that the patient met the eligibility criteria and details of medical history were recorded. Information regarding comorbidities, particularly cardiovascular diseases, diabetes and liver or renal failure was collected. A forced spirometry was performed following criteria of the Spanish Society of Pneumology and Thoracic Surgery [14] and sputum samples were obtained. Patients unable to produce sputum were susceptible to reassessment for airway colonisation at least one month after the initial investigation for a maximum of three consecutive visits.

Microbiological sputum study

A sputum sample was obtained and processed within 60 minutes on the day of the visit according to standard methods [13,15,16]. Patients who did not produce sputum spontaneously underwent sputum induction. In brief, patients were pretreated with an inhaled β_2-agonist ten minutes before the nebulisation of isotonic saline (0.9%) with an ultrasonic nebuliser (Ultraneb2000, DeVilbiss Healthcare Inc., Somerset, PA, USA), that was followed by increasing concentrations of hypertonic saline (3%, 4% and 5%), for 7 min with each concentration. After every induction, the patient attempted to obtain a sputum sample by coughing, and the nebulisation procedure was stopped when the sputum volume collected was 1 mL or more [17]. In current smokers, sputum induction was performed after at least 6 hours of tobacco abstinence. The purulence of sputum was graded in a scale from 1 to 5 according to the colour from white -1- to greenish -5-, always by the same researcher at each centre. The sample was weighed and processed with a 4-fold volume of dithiothreitol (Sputasol, Oxoid Ltd., Hants, UK) and was cultured. Sputum samples were serially diluted and plated on chocolate agar enriched, chocolate agar with bacitracin, *Haemophilus*-selective agar, blood agar, and McConkey agar. Plates were incubated for 24-48 hours at 37°C and in 5% CO_2 atmosphere. Microorganisms were identified by colony morphology, Gram staining and specific culture conditions (e.g., requirements for factors for growth, presence of oxidase and catalase, porphyrin synthesis). Cultures were considered positive for bronchial colonisation if microorganisms considered as PPMs such as *Haemophilus influenzae, Haemophilus parainfluenzae, Streptococcus pneumoniae, Moraxella catarrhalis, Pseudomonas aeruginosa*, enterobacteria and/or *Staphylococcus aureus* were grown at loads of at least 100 colony-forming units (CFU)/mL according to previously defined criteria [18,19]. Colonised patients were then divided into high (>10^5 CFU/mL) or low (≤10^5 CFU/mL) bacterial load according to previous studies [4,8].

Sputum concentrations of pro-inflammatory cytokines, including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor-alpha (TNF-alpha) were measured using quantitative sandwich immunoassay techniques in processed supernatants as previously described [20].

Statistical analysis

Variables were presented as mean values and standard deviations, those not following a normal distribution were presented as median and interquartile range (IQR,

25th-75th percentile). Categorical variables were compared with the chi-square test and continuous variables with the Student's *t* test or the Mann-Whitney U test when data departed from normality. Following univariate analysis, variables were included in two stepwise logistic regression models constructed as exploratory analysis to identify independent risk factors for bronchial colonisation and factors significantly associated with high bacterial load as opposed to low bacterial load and sterile sputum cultures. The variables included in the models were: age, gender, active versus ex-smoker, pack-years of smoking, FEV_1 (% predicted), degree of dyspnoea, colour of sputum, cardiovascular comorbidity and number of exacerbations and hospitalisations the previous year. Bilateral two-tailed hypotheses were formulated and 95% confidence intervals (CI) were calculated. Statistical significance was set at $P < 0.05$.

Results

A total of 119 patients (92.5% men) with a mean (standard deviation, SD) age of 68.1 (9.1) years were studied. The clinical characteristics of these patients are reported in Table 1. Induction of sputum was necessary to obtain a valid sputum sample in only 5 cases (3 in one centre and 2 in the other). Bacterial colonisation was demonstrated in 58 (48.7%) patients, 2 in samples obtained by sputum induction. Results of sputum microbiology are shown in Table 2. Colonisation by a single PPM was recorded in 50 patients. Eight subjects yielded more than one PPM in their sputum. *Haemophilus influenzae* and *H. parainfluenzae* made up 72% of all bacterial isolates.

Table 1: Clinical characteristics of the study population

Data	Frequency
Subjects, no.	119
Sex, men, no. (%)	112 (92.5)
Age, years, mean (SD)	68.1 (9.1)
Current smokers, no. (%)	11 (9.2)
Smoking, pack-years, mean (SD)	40 (21.1)
Cardiovascular morbidity, no. (%)	36 (29.7)
Exacerbations in the previous year, mean (SD)	1.3 (0.5)
Requiring hospital admission	0.3 (0.5)
Post-bronchodilator spirometry, mean (SD)	
FVC, mL	2790 (942)
FVC, %	68.9 (19.2)
FEV_1, mL	1406 (493)
FEV_1, %	46.4 (14.1)

Table 2: Potentially Pathogenic Microorganisms (PPMs) isolated in colonised COPD patients.

	No. (%)
Microorganisms isolated	
Haemophilus influenzae	21 (42)
Haemophilus parainfluenzae	15 (30)
Pseudomonas aeruginosa	5 (10)
Streptococcus pneumoniae	4 (8)
Moraxella catarrhalis	4 (8)
Staphylococcus aureus	1 (2)
Mixed colonisations (from the above microorganisms)	
H. influenzae + *S. pneumoniae*	1
H. influenzae + *P. aeruginosa*	3
H. influenzae + *H. parainfluenzae*	2
P. aeruginosa + *S. viridans*	2

There were significant differences in cigarette consumption, cough, dyspnoea, comorbidities, COPD exacerbations and hospitalisations in the previous year, and sputum colour between patients with and without bacterial colonisation (Table 3).

The distribution of colonised patients according to sputum colour is presented in Figure 1. Samples with colour 1 (white) were predominantly sterile, whereas in the samples with colours 3 to 5 (yellow to greenish) the prevalence of colonisation was higher than 80%. Colour number two (light yellow) was not discriminative between colonised and non-colonised.

When colonised patients were divided according to bacterial load, 36 patients had a high bacterial load ($>10^5$ CFU/mL) and the remaining 22 had a low bacterial load ($\leq 10^5$ CFU/mL). The characteristics of colonised patients with a high bacterial load (n = 36) were compared with a group formed by non-colonised patients (n = 61) and those with a low bacterial load (n = 22) considered together (n = 83). Statistically significant differences between the two groups in smoking (pack-years), cough, grade of dyspnoea, hospitalisations in the previous year and sputum colour persisted when patients with high bacterial loads were compared with the remaining

Table 3: Differences between stable COPD patients with and without bacterial colonisation

Variables	Colonised (n = 58)	Not colonised (n = 61)	*P* value
Sex, men, no. (%)	54 (93.1)	55 (90.2)	0.74
Age, years, mean (SD)	68.3 (8.3)	67.6 (9.8)	0.67
Current smokers, no. (%)	7 (12.1)	4 (6.6)	0.35
Smoking, pack-years, mean (SD)	46.7 (25.1)	34.2 (23.4)	0.006
Cardiovascular morbidity, no. (%)	22 (37.9)	20 (32.8)	0.23
Comorbid conditions, mean (SD)	1.06 (0.99)	0.61 (1.02)	0.025
Use of inhaled steroids, no. (%)	46 (79.3)	47 (77.1)	0.83
Symptoms, no. (%)			
Dyspnoea	56 (96.5)	58 (95.1)	0.72
Cough	44 (75.9)	56 (91.8)	0.024
Expectoration	57 (98.3)	58 (95.1)	0.62
Grade of dyspnoea, mean (SD)	1.78 (0.92)	1.15 (0.54)	<0.001
Exacerbations in the previous year, no. (%)			
Number			0.021
≤2	31 (53.4)	47 (77.1)	
>2	27 (46.6)	14 (22.9)	
Requiring hospital admission			0.007
None	36 (62.1)	51 (83.6)	
≤1	16 (27.6)	10 (16.4)	
>1	6 (10.3)	0	
Lung function tests, mean (SD)			
FVC, mL	2852.7 (979.1)	2710.9 (911.5)	0.41
FVC, %	70.5 (19.5)	66.7 (18.9)	0.28
FEV_1, mL	1411.4 (511.7)	1380.0 (433.1)	0.72
FEV_1, %	47.4 (15.2)	45.1 (13.1)	0.38
FEV_1/FVC	50.4 (11.8)	52.6 (13.9)	0.43
Sputum analysis			
Colour, mean (SD)	2.94 (1.0)	1.56 (0.8)	<0.001
Pro-inflammatory cytokines, median (IQR) in pg/mL			
IL-1, n = 53	14 (4-432)	168 (49-758)	0.82
IL-6, n = 53	258 (76-653)	112 (33-368)	0.62
IL-8, n = 61	13480 (1335-43400)	5390 (252-14335)	0.27
TNF-alpha, n = 54	45 (20-94)	35 (10-183)	0.12

FVC = forced vital capacity; FEV_1 = forced expiratory volume in the first second; IQR = interquartile range; IL= interleukin; TNF = tumour necrosis factor.

patients (Table 4). Sufficient sputum for inflammatory analysis was available from only 61 subjects, all from spontaneous sputum. Sputum concentrations of inflammatory markers showed a great inter-individual variability and did not follow a normal distribution. There were no significant differences in sputum concentrations for any of the inflammatory markers analysed between patients with or without bacterial colonisation (Table 3). The lack of significance persisted when patients with high bacterial load were compared with those with low bacterial load and not colonised. However, in this last comparison, patients with high bacterial load presented consistently (but not significantly) higher concentrations of all pro-inflammatory cytokines except IL-6 (Table 4).

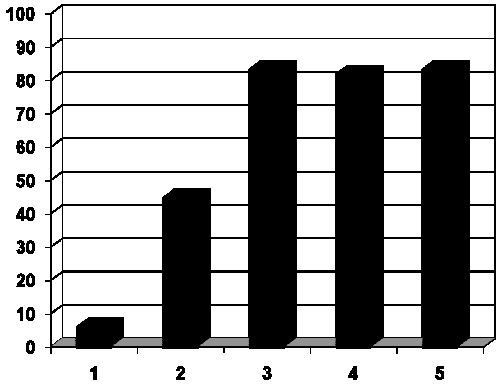

Figure 1 Percentage of bacterial colonisation according to sputum colour (differences statistically significant at $P < 0.001$).

The results of the multivariate analysis were very similar when identifying the factors significantly associated with the presence of PPMs or on classifying the population according to bacterial load. In both cases, only the degree of dyspnoea and sputum colour were significantly and independently associated with the presence of PPMs and with high bacterial load. Sputum colour was a stronger indicator of the presence of positive cultures for PPMs than its load (Table 5).

Discussion

In the present study, bacterial colonisation of the airways by PPMs, mainly *H. influenzae* and *H. parainfluenzae*, was reported in 49% of patients with stable COPD. This finding adds evidence to a high prevalence of bacterial colonisation of airways in stable COPD reported by others [4,5,9-12]. Interestingly, our results using sputum samples are quite similar to those obtained in other studies with the use of the PSB technique or bronchial lavage for microbiologic assessment of the lower airways in COPD [4,5,11,12,20,21]. The possibility of sputum collection along a maximum of three monthly clinical visits and the use of the induced sputum technique in selected cases may have accounted for this high diagnostic yield of the sputum. However, most of our patients were able to produce a valid sputum sample for microbiological examination and induction of sputum was necessary in only 5 cases. A previous study by our group demonstrated that spontaneous and induced sputum yielded equivalent results in terms of frequency of bacterial colonisation and species recovered [22]. A pooled analysis of data from studies that used PSB demonstrated that a PPM load $\geq 10^2$ CFU/mL should be considered abnormal and allowed the estimation that at least one quarter of the patients with stable COPD were colonised by PPMs [5]. Furthermore, most patients with exacerbated COPD had concentration of PPMs > 10^5 [4,5]. Since there is no universally accepted cut-off for high bacterial load in sputum samples, a 10^5 CFU/mL concentration was used in our study [4,8]. With this value, 30% of our total population and almost two thirds of the colonised patients in our study had a high PPM load.

Bacterial colonisation in our study was related to cumulative consumption of cigarette smoking, history of exacerbations in the previous year and sputum colour. Exacerbations in the previous year leading to hospitalisation were associated with increased bacterial load, although this relationship disappeared on multivariate analysis. In other studies, current smoking and severe airflow obstruction have been identified as predisposing factors for bacterial colonisation in stable COPD [11,12]. However, we did not observe significant differences in lung function between colonised and non-colonised patients. The relationship between lung function and frequency of colonisation is not clear, since a lack of association between FEV_1 and colonisation has also been observed in other studies [8,12,21,23] and may be due, at least in part, to the under-representation of mild patients in most series as well as in the current study. Interestingly, the only two factors identified in multivariate analysis to be significantly and independently associated with both presence of bacterial colonisation and high bacterial load were a more severe degree of dyspnoea and a darker colour of sputum. The degree of dyspnoea is a marker of severity of COPD and being a categorical variable with a wider distribution in our population probably contributed to its demonstrated association with colonisation, in contrast to the severity of FEV_1 impairment.

Regarding bronchial inflammation, it should be noted that we did not find increased sputum concentrations of pro-inflammatory cytokines in patients with bacterial colonisation. Different reasons may explain this finding, including a small number of patients with valid samples for analysis, the inter-individual variability in the sputum concentrations of the cytokines was very large [24], and there was a large number of patients with low bacterial loads. In fact, Hill *et al*. [8] have demonstrated that markers of inflammation increased progressively with increasing bacterial load in patients with stable COPD. Consequently, when our colonised patients were categorized according to high or low bacterial load, besides the persistence of the clinical differences already observed between the colonised and non-colonised groups (i.e., cigarette smoking, hospitalisations in the previous year, grade of dyspnoea and sputum colour) a non-significant trend towards higher sputum concentrations of inflammatory markers (except IL-6) was observed in patients with high bacterial load. Our results concur with previous observations regarding the lack of association

Table 4: Differences between colonised and non-colonised COPD patients according to bacterial load

Variables	High bacterial load (≥10^5) (n = 36)	Low bacterial load (<10^5) and not colonised (n = 83)	*P* value
Sex, men, no. (%)	33 (91.7)	76 (91.6)	0.98
Age, years, mean (SD)	68.6 (6.9)	67.7 (9.9)	0.63
Current smokers, no. (%)	6 (16.7)	5 (6)	0.086
Smoking, pack-years, mean (SD)	48.5 (22.5)	36.7 (25.2)	0.017
Cardiovascular morbidity, no. (%)	11 (30.6)	31 (37.3)	0.34
Comorbid conditions, mean (SD)	0.86 (0.99)	0.83 (1.02)	0.88
Use of inhaled steroids, no (%)	29 (80.6)	64 (77.1)	0.81
Symptoms, no. (%)			
Dyspnoea	36 (100)	78 (93.9)	0.66
Cough	25 (69.4)	75 (90.4)	0.007
Expectoration	36 (100)	79 (95.2)	0.31
Grade of dyspnoea, mean (SD)	1.86 (0.83)	1.28 (0.73)	<0.001
Exacerbations in the previous year, no. (%)			
Number			0.32
≤2	20 (55.6)	58 (69.9)	
>2	16 (44.4)	25 (30.1)	
Requiring hospital admission			0.003
None	20 (55.6)	67 (80.7)	
≤1	11 (30.6)	15 (18.1)	
>1	5 (13.9)	1 (1.2)	
Lung function tests, mean (SD)			
FVC, mL	2936.9 (975.7)	2712 (927.6)	0.23
FVC, %	71.9 (21.0)	67.1 (18.4)	0.21
FEV_1, mL	1423.9 (536.5)	1382 (443.1)	0.66
FEV_1, %	47.3 (15.9)	45.8 (13.4)	0.61
FEV_1/FVC	48.9 (11.2)	52.7 (13.5)	0.15
Sputum analysis			
Colour, mean (SD)	2.97 (0.94)	2.01 (1.08)	<0.001
Pro-inflammatory cytokines, median (IQR) in pg/mL			
IL-1, n = 53	47 (5-593)	29 (4-255)	0.14
IL-6, n = 53	134 (39-381)	169 (34-415)	0.18
IL-8, n = 61	8060 (460-31400)	4890 (201-15025)	0.09
TNF-alpha, n = 54	76 (11-269)	38 (11-71)	0.96

FVC = forced vital capacity; FEV_1 = forced expiratory volume in the first second; IQR = interquartile range; IL= interleukin; TNF = tumour necrosis factor.

Table 5: Results of multivariate analysis of factors associated with presence of bacteria in sputum and with high bacterial load.

Factor	OR	95% CI	*P* value
Factors associated with bacteria in sputum			
Degree of dyspnoea	2.63	1.53 - 5.09	0.004
Sputum colour	4.11	2.30 - 7.29	<0.001
Factors associated with high bacterial load as opposed to no bacteria and low bacterial load			
Degree of dyspnoea	2.01	1.17 - 3.46	0.012
Sputum colour	1.99	1.32 - 2.99	0.001

between colonisation and increased IL-6 [9,10] but are discordant with other works showing significantly increased bronchial IL-8 and TNF-alpha in colonised patients, particularly with *H.influenzae* [9,10,21,23,25]. Therefore, our data, if confirmed in a larger sample of patients, would also suggest a dose-response relationship between bacterial load and bronchial inflammation and that a threshold of bacterial load might be necessary to elicit a significant inflammatory reaction in the airways [5,6,26]. In contrast, Sehti *et al*. [27] examined whether the increase in bacterial concentrations functions as a separate mechanism of exacerbation induction, independent of a new strain acquisition. In a prospective longitudinal cohort of COPD patients assessed during exacerbations and stable disease, sputum concentrations of pre-existing strains of *H. influenzae* and *H. haemolyticus* were not significantly different in exacerbation versus stable disease. Concentrations of *M. catarrhalis* and *S. pneumoniae* were even lower during exacerbations compared with stable periods. However, concentrations of new strains of *H. influenzae* and *M. catarrhalis* were increased during exacerbations, but the differences were small. These authors speculate that change in bacterial load was unlikely to be a major primary mechanism of exacerbation induction in COPD [27,28]. This hypothesis is a matter of debate, because the interpretation of what a significant increase in bacterial load is when measured in a logarithmic scale is not clear [10], and when transformed to a non-logarithmic scale, the differences in absolute bacterial counts were of a very high magnitude [29].

The identification of bronchial colonisation has clinical implications. Patel *et al*. [9] demonstrated that the presence of lower airway bacterial colonisation in stable COPD was significantly related to exacerbation frequency and severity. In the study of Rosell *et al*. [5], again high bacterial loads were associated with exacerbation and showed a statistically significant dose-response relationship between bacterial load and exacerbation after adjustment for covariates. In our study colonised patients had significantly more exacerbations and hospital admissions the year previous to the study compared with non-colonised patients, but the significance disappeared on multivariate analysis. It should be taken into account that our study was neither designed nor powered to demonstrate differences in exacerbation or hospitalisation rates between colonised and non-colonised COPD patients. Therefore, the identification of patients colonised by PPMs using a non-invasive and relatively inexpensive technique such as the analysis of sputum may play an important role in the management of severe and very severe COPD, particularly if intervention studies with antibiotics demonstrate improved clinical outcomes [13].

To facilitate the diagnosis of bronchial colonisation the use of a surrogate marker could be of interest. Purulence (colour) of sputum graded by the investigator with a simple scale from 1 to 5 revealed significant differences in colour between colonised and non-colonised patients. Patients with colour 3 or higher (dark yellow to green sputum) had a prevalence of bacterial colonisation greater than 80%. The relevance of sputum colour has been already described and validated for exacerbated patients in which yellowish or greenish sputum is significantly associated with a bacterial exacerbation compared with white (non-bacterial) sputum [30,31] but the relationship between sputum colour and bacterial colonisation in stable COPD has deserved little attention [8].

The present results should be interpreted taking into account some limitations of the study, particularly the small sample size may not have allowed determination of sputum concentrations of inflammatory markers in all samples, in most cases due to the small recovery of sputum that did not provide enough supernatant for the quantification of inflammatory mediators. The cross-sectional design did not allow the dynamics and time course of bacterial colonisation and airway inflammation during exacerbations to be examined. Patients with negative bronchodilator test were included to exclude individuals with asthma who are less likely to be colonised, but the results may not be extrapolated to partially reversible COPD patients. High concentrations of PPMs in sputum samples, however, is a simple parameter that may help to

select candidates to participate in antibiotic trials of stable COPD in order to demonstrate bacterial eradication and potentially prolong time to exacerbation [6,32,33].

Conclusions

Almost half of a population of ambulatory moderate to very severe COPD patients carry PPMs in their airways. Colonised patients had more severe dyspnoea, and sputum colour allows the identification of patients most likely to be colonised by PPMs.

List of abbreviations

CFU: colony-forming units; CI: confidence interval; COPD: Chronic obstructive pulmonary disease; CT: computed tomography; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity; IQR: interquartile range; IL-1: interleukin-1; IL-6: interleukin-6; IL-8: interleukin-8; OR: odds ratio; PPMs: potentially pathogenic microorganisms; PSB: protected specimen brush; SD: standard deviation; TNF-alpha: tumour necrosis factor-alpha.

Competing interests

Marc Miravitlles has received honoraria for consultancy and speaking at scientific meetings from Bayer Schering, GlaxoSmithKline, Boehringer Ingelheim and AstraZeneca. Cristian de la Roza is fully employed in the Medical Department of Bayer Schering Pharma. Antoni Torres has received honoraria for consultancy and speaking at scientific meetings from Bayer and Covidien. Alicia Marín, Eduard Monsó, Sara Vilà, Ramona Hervás, Cristina Esquinas, Marian García, Laura Millares and Josep Morera have no conflict of interest to disclose.

Authors' contributions

MM designed the study, participated in the analysis and interpretation of data and wrote the manuscript. EM, JM and AT designed the study, and participated in the analysis and interpretation of data. AM and SV recruited the patients, collected data and participate in the design and analysis. CR participated in the design and analysis of the study. CE and RH collected and processed the samples, and created and cleaned the database. LM and MG perfomed the microbiological investigations. All authors read and approved the final manuscript.

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4. Estudi 4: *Efficacy of moxifloxacin in the treatment of bronchial colonisation in COPD*

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Resum

Aquest estudi va ser dissenyat per investigar l'eficàcia de moxifloxací per a l'eradicació de la colonització bacteriana de les vies aèries en pacients amb MPOC moderada-greu.

Dels 119 pacients estudiats, 40 (edat mitja de 69 anys, VEMS mig del 50% del predit) estaven colonitzats per microorganismes potencialment patògens i van ser inclosos en un assaig clínic controlat doble cec amb moxifloxací 400 mg /dia durant 5 dies.

La taxa d'eradicació fou dels 75% amb moxifloxací i del 30% amb placebo a les 2 setmanes (p=0.01). La persistència de colonització bacteriana a les 8 setmanes a la branca placebo va ser elevada encara que no significativa (5 pacients de 20 (25%) en comparació amb 1 de 20 (5%) en la branca moxifloxací). Les freqüències d'adquisició d'una nova soca bacteriana varen ser similars en ambdós grups de tractament, per la qual cosa la prevalença de colonització a les 8 setmanes va ser equivalent. No es varen trobar diferències en el nombre de pacients que havien patit una exacerbació als 5 mesos de seguiment. Només l'adquisició d'una nova soca bacteriana durant el seguiment va mostrat una relació estadísticament significativa amb l'aparició d'una exacerbació.

moxifloxací va ser efectiu eradicant microorganismes potencialment patògens en pacients amb cultius d'esput positius, però molts pacients presentaren recolonització a les 8 setmanes de seguiment. L'adquisició d'una nova soca bacteriana estava associada amb un increment del risc de patir una exacerbació.

Resultats

Es van avaluar un total de 119 pacients dels quals 92.5% eren homes, amb una mitjana de edat de 68 anys i una mitjana de VEMS de 46% del predit. Amb la tècnica de l'esput induït, 45 (37.8%) pacients van produir un esput amb cultiu positiu. En 5 casos el cultiu d'esput va ser positiu per a *Pseudomonas aeruginosa, fet pel qual aquests* pacients es van excloure de l'estudi. Els 40 pacients restants amb cultiu positiu per altres patògens van ser aleatoritzats en dos grups a rebre placebo (n=20) o moxifloxací 400 mg/dia durant 5 dies (n=20). Els dos grups presentaven característiques sociodemogràfiques, respiratòries i microbiològiques semblants, excepte per un increment en la freqüència d'exacerbacions l'any previ en el grup tractat amb moxifloxací.

A la visita de les 2 setmanes posteriors a la randomització, 15 (75%) pacients del grup tractat amb moxifloxací mostraven cultius d'esput negatius en front a 6 (30%) pacients del grup tractat amb placebo (p=0.01). En el grup tractat amb placebo, dels 14 casos amb aïllament a l'esput de MPPs, 10 presentaven cultius d'esput positius i 4 eren negatius però amb una determinació de PCR positiva seguida de cultius positius pel mateix microorganisme (*Haemophilus influenzae*) a les 8 setmanes de l'aleatorització. Aquests 4 microorganismes mostraven el mateix perfil en el tipatge molecular des de la visita basal fins a la setmana 8, confirmant-se la persistència del patogen inicial en el grup placebo i els resultats falsos negatius del cultiu d'esput a la visita 2. Les determinacions de PCR en els cultius d'esput negatius a les 2 setmanes al grup tractat amb moxifloxací van ser negatives en tots els casos.

A les 8 setmanes després de la randomització, la persistència de colonització bacteriana a la branca placebo va ser elevada respecte al grup moxifloxací, encara que no significativa (5 pacients de 20 (25%) en comparació amb 1 de 20 (5%) en la branca moxifloxací; p=0.18). Les freqüències d'adquisició d'una nova soca bacteriana varen ser similars en ambdós grups de tractament (placebo 60%

versus moxifloxací 70%; p>0.25), per la qual cosa la prevalença de colonització a les 8 setmanes també va ser similar (80% en el grup placebo i 75% en el grup moxifloxací; p>0.25).

No es varen trobar diferències en el nombre de pacients que havien patit una exacerbació als 5 mesos de seguiment: 6 (30%) en el grup moxifloxací i 5 (25%) en el grup placebo; p>0.25). El temps fins a la primera exacerbació tampoc diferia entre ambdós grups (74±39 dies *versus* 60±39 dies per al grup placebo i moxifloxací respectivament; p>0.25).

Ajustant per edat i VEMS post broncodilatador en el model de regressió logística, ni el tractament amb moxifloxací ni la presència de microorganismes persistents en els cultius d'esput durant el seguiment, es van associar amb l' aparició d'una exacerbació. Només l'adquisició d'una nova soca bacteriana durant el seguiment va mostrar una relació estadísticament significativa amb l'aparició d'una exacerbació (OR 9.63, 95% CI 1.01-91,64).

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Efficacy of moxifloxacin in the treatment of bronchial colonisation in COPD

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ABSTRACT: This study was designed to investigate the efficacy of moxifloxacin for the eradication of bacterial colonisation of the airways in patients with moderate-to-severe chronic obstructive pulmonary disease (COPD).

Out of 119 stable patients with COPD screened, 40 (mean age 69 yrs, mean forced expiratory volume in 1 s 50% predicted) were colonised with potentially pathogenic microorganisms (PPMs) and were included in a randomised, double-blind, placebo-controlled trial with moxifloxacin 400 mg daily for 5 days.

Eradication rates were 75% with moxifloxacin and 30% with placebo at 2 weeks ($p=0.01$). Bacterial persistence at 8 weeks was still higher (not significantly) in the placebo arm (five (25%) out of 20 *versus* one (5%) out of 20; $p=0.18$). The frequencies of acquisition of a new PPM were high and similar in both treatment groups; consequently, the prevalence of colonisation at 8 weeks was also similar between treatment arms. No difference was found in the number of patients with exacerbations during the 5-month follow-up. Only the acquisition of a new PPM during follow-up showed a statistically significant relationship with occurrence of an exacerbation.

Moxifloxacin was effective in eradicating PPMs in patients with positive sputum cultures. However, most patients were recolonised after 8 weeks of follow-up. Acquisition of a new strain of bacteria was associated with an increased risk of developing an exacerbation.

KEYWORDS: Antibiotics, bacterial colonisation, chronic obstructive pulmonary disease, moxifloxacin

Bronchial bacterial colonisation is frequent in patients with chronic obstructive pulmonary disease (COPD) [1–3]. Studies performed using the protected specimen brush have demonstrated that up to 45% of patients with moderate-to-severe COPD carry potentially pathogenic microorganisms (PPMs) in significant concentrations in their lower airways [4]. The clinical significance of bacterial colonisation of the airways has recently been recognised. Colonised patients have increased lower airway [5–7] and systemic inflammation [8], present more frequent and severe exacerbations [9] and the changes in bacterial load and species are associated with a steeper decline in lung function over time [10]. Furthermore, one study has observed that colonised patients had more severely impaired health-related quality of life than patients whose airways were sterile [8].

Understanding the role of bacterial colonisation in COPD is crucial for the development of strategies aimed at eliminating the PPMs from the lower airways [11]. The new, more active antibiotics may allow short courses of treatment to be administered with the objective of eradicating bacteria in the airways. In this respect, moxifloxacin is a fourth generation fluoroquinolone that has demonstrated bactericidal activity against the most frequently isolated PPMs in respiratory secretions in patients with COPD [12, 13] and clinical efficacy in the treatment of exacerbations of COPD [14, 15]. Therefore, moxifloxacin is indicated in most guidelines for the treatment of exacerbations of COPD in patients with moderate-to-severe disease or with risk factors for failure [16]. The aim of the current study was to investigate the efficacy of moxifloxacin in eradication of PPMs in stable patients with COPD and the persistence of eradication over a period of 8 weeks after treatment.

METHODS

Patients

Patients $\geqslant$40 yrs of age with COPD were recruited between January 2006 and April 2007 in two centres in the Barcelona Metropolitan Area

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in Spain. Inclusion criteria were as follows: smoking history of $\geqslant$10 pack-yrs, post-bronchodilator forced expiratory volume in 1 s (FEV1) <60% predicted; an increase of FEV1 with the use of 400 μg of salbutamol of <10% pred for that patient; and a ratio of post-bronchodilator FEV1 to forced vital capacity $\leqslant$0.70 [17]. A positive sputum culture for PPMs at a concentration of $\geqslant 10^2$ colony-forming units (CFU)·mL^{-1} at the screening visit was required for inclusion. According to CABELLO *et al.* [18], PPMs were defined as those microorganisms recognised as agents causing respiratory infections, regardless of whether they belonged to the gastrointestinal or oropharyngeal flora: Gram-negative rods, such as Enterobacteriaceae and *Haemophilus* spp.; Gram-positive cocci, such as *Staphylococcus aureus, Streptococcus pneumoniae*; and Gram-negative cocci, such as *Moraxella catarrhalis*. Exclusion criteria were: diagnosis of asthma; any respiratory disorder other than COPD; exacerbation or use of parenteral or oral steroids or change in COPD medication in the 4 weeks before randomisation; and a course of antibiotics or hospitalisation in the 16 weeks before randomisation. Other exclusion criteria were: colonisation by *Pseudomonas aeruginosa* or any contraindication for the use of fluoroquinolones.

All patients gave written informed consent and the study protocol was approved by the Spanish Agency of Medicinal Products (Agencia Española del Medicamento y Productos Sanitarios; number 04-0112). Institutional review boards of both participating centres also approved the study and it was conducted according to the good clinical practice guidelines and the last version of the Declaration of Helsinki.

Study design

This was a randomised, double-blind, parallel-group, placebo-controlled trial. Patients fulfilling inclusion and exclusion criteria were screened for bronchial colonisation by PPMs in sputum. Those with sputum samples negative for PPMs were rescheduled for up to three consecutive visits at 2-week intervals to obtain a positive sputum culture. Patients with a positive sample were randomised 1:1 to receive moxifloxacin (Actira®; Bayer AG, Leverkusen, Germany) 400 mg or placebo, once daily for 5 days. Active medication and matching placebo were provided by Bayer Pharma (Barcelona, Spain) and were stored and distributed by the Pharmacy Department of the Hospital Clínic (Barcelona, Spain), which also developed the randomisation tables for the study. Follow-up visits were scheduled 2 and 8 weeks after randomisation, when clinical assessment and microbiology of sputum were investigated.

Episodes of exacerbations, defined as acute episodes of increased dyspnoea, sputum production and/or purulence treated with antibiotics and/or oral corticosteroids [19], and appearing during up to 5 months of follow-up, were recorded.

Measurements

The primary efficacy end-point was the rate of eradication at visit two, 2 weeks after randomisation. Secondary end-points were rate of eradication and frequency of acquisition of new PPMs at visit three (8 weeks after randomisation), and frequency of exacerbations at 5 months of follow-up.

At randomisation, sociodemographic data, cardiovascular comorbidity, smoking habits, respiratory symptoms, and number of previous exacerbations and hospitalisations were collected. The microbiological characteristics of the sputum were recorded and all subjects performed forced spirometry and reversibility tests in the morning with the same dry rolling-seal spirometer (Spirometrics, Gray, ME, USA), according to standard techniques [20].

Sputum sampling and microbiological analysis

The sputum sample was processed within 60 min of collection according to standard methods [21, 22]. Briefly, the patient was pretreated with an inhaled β_2-agonist 10 min before the nebulisation of isotonic saline (0.9%) followed by increasing concentrations of hypertonic saline (3%, 4% and 5%), for 7 min with each concentration. After every induction, the patient attempted to obtain a sputum sample by coughing, and the nebulisation procedure was discontinued when the sputum volume collected was $\geqslant$1 mL [23]. In current smokers, sputum induction was performed after $\geqslant$6 h of tobacco abstinence. Sputum appearance was determined according to the Murray–Washington (MW) criteria [24] and samples with >25 leucocytes per field (MW $\geqslant$3) were considered indicative of a neutrophilic inflammatory response [25]. The sample was weighed and processed with a four-fold volume of dithiothreitol (Sputasol; Oxoid Ltd, Basingstoke, UK) and cultured. The determination of microbial typology and load was carried out by culture in selective media and serial dilutions, according to standard methods [26, 27]. For the purpose of this study, only cultures growing PPMs, such as *Haemophilus influenzae, Haemophilus parainfluenzae*, *M. catarrhalis*, *S. pneumoniae*, enterobacteria and/or *S. aureus* were considered positive, according to previously defined criteria [18, 28, 29]. Quantitative cultures were expressed as CFU·mL^{-1}.

Detection of microorganism DNA by PCR

Supernatant was recovered after sputum centrifugation at $500 \times g$ for 10 min and stored at -70°C for PCR analyses. For each PPM recovered, six colonies were stored at -70°C for posterior molecular typing. DNA extraction was performed from sputum samples with negative cultures at the 2- or 8-week visit in order to investigate the presence of PPM DNA using a QIAamp DNA mini kit (Quiagen, Hilden, Germany). Briefly, two pairs of specific oligonucleotide primers for the p6 gene of *H. influenzae* and *H. parainfluenzae* were designed using Primer Express software (Applied Biosystems, Foster City, CA, USA) and synthesised (Roche Diagnostics, Indianapolis, IN, USA). Since the p6 gene sequences of the *H. influenzae* and *H. parainfluenzae* differ in 30 of 274 pb, it is possible to design specific primers for each species. Oligonucleotide sequences were forward primer 5′-AATTTCCAGCTTGGTCTCCA-3′ and reverse 5′-CAAAAGTTTGAGCAGCACCA-3′ for *H. influenzae*, forward primer 5′-CCGTTTACTTCGGTTTCGAC-3′ and reverse 5′- CAGCACGACGTTGACCTAAA-3′ for *H. parainfluenzae*, and detection, forward primer 5′-GTGAGTGCC-GCTTTTATAACC-3′ and reverse 5′-TGTATCGCCTGCCAA-GACAA-3′ for *M. catarrhalis*, as previously described by GREINER *et al* [30]. A total of 10 μL of the PCR product underwent electrophoresis in 2% agarose in Tris/borate/EDTA buffer and visualised by ethidium bromide staining. The sizes of the amplified products, 167 bp, 171 bp and 71 bp, for *H. influenzae*, *H. parainfluenzae* and *M. catarrhalis* respectively, were compared with those of a positive control

amplified product and a molecular mass marker ladder (Promega, Madison, WI, USA). Samples showing PPM DNA with a posterior positive culture for the same PPM in the following 3 months were considered as colonised by the PPM in spite of the negative sputum culture.

Molecular typing of PPMs

Molecular PPM typing was performed by pulse field electrophoresis to determine whether a PPM recovered from consecutive samples corresponded to the persistence of the same strain or to the acquisition of a new one [31]. Briefly, chromosomal DNA from multiple (>6) PPMs was extracted in agarose plugs. After enzyme digestion (New England Biolabs, Ipswich, MA, USA), restriction fragments were separated using a contour-clamped homogeneous electric field system (CHEF DR II; BioRad, Ivey sur Seine, France) at 14°C and 5 $V \cdot cm^{-1}$, beginning with an initial 5.6-s pulse which was linearly increased until 40.6 s and maintained for 25 h in 0.5 x Tris/borate/EDTA (Sigma Chemical, St Louis, MO, USA). Bacteriophage lambda concatemer (New England Biolabs, Ipswich, MA, USA) was included as the molecular weight DNA marker. The patterns obtained were analysed using the Diversity Database Software (BioRad). PPMs cultured from follow-up sputum samples showing the same molecular profile by pulse field electrophoresis as those previously recovered were considered as persistent strains, whereas PPMs cultured at follow-up, which were not present in the previous sample, were considered as acquired strains.

Statistical analysis

All data were analysed using the SPSS statistical software package version 15 (Chicago, IL, USA). Results were expressed as absolute and relative frequencies for categorical variables and for continuous variables as mean±SD, or median (interquartile range) when they did not follow a normal distribution. Sputum characteristics at 2 and 8 weeks after randomisation were compared between the two groups for the frequencies of PPM eradication, persistence and acquisition during follow-up and the effect of moxifloxacin treatment on these parameters (Chi-squared, Fisher exact, unpaired t-test and Mann–Whitney U-test, when appropriate). Finally, the frequency of exacerbations in both groups up to 5 months after randomisation was compared (Chi-squared, Fisher exact and unpaired t-test), and the determinants of exacerbation were calculated by logistic regression, expressing the results as odds ratios and 95% confidence intervals, considering moxifloxacin treatment, PPM persistence and acquisition as independent variables, and adjusting the models for age and lung function (post-bronchodilator FEV_1 % pred). A p-value $\leqslant 0.05$ was reported as statistically significant.

RESULTS

A total of 119 consecutive stable COPD outpatients accepted to participate in the study and were screened for bronchial colonisation by PPMs. The mean age was 68±9.1 yrs, 110 were males and the mean post-bronchodilator FEV_1 was 46.2±14.1% pred. Using induced sputum, 45 (37.8%) patients provided a positive sputum culture; this was positive for *P. aeruginosa* in five patients, who were excluded from the study. The remaining 40 patients with positive sputum cultures for PPMs other than *P. aeruginosa* were randomised to receive either placebo (n=20) or moxifloxacin (n=20). The socio-demographic, respiratory and microbiological profiles were comparable except for a higher exacerbation frequency in the previous year in patients treated with moxifloxacin (table 1).

On the visit week two after randomisation, PPM isolation from sputum became negative in 15 (75%) out of 20 patients treated with moxifloxacin and in six (30%) out of 20 who received placebo (p=0.01). Assuming a one-side contrast of hypothesis and accepting an α risk of 0.05, the sample size of 20 subjects per group provides a power of 92% to identify as statistically significant the observed difference in eradication of 75% *versus* 30%. In the placebo-treated group, the 14 cases with positive isolation of PPM consisted of 10 cases with positive sputum cultures and four cases of negative cultures but positive PCR testing followed by a positive sputum culture for the same microorganism (*H. influenzae*) at 8 weeks after randomisation. Furthermore, these four PPMs showed the same molecular typing profile throughout the study, from the baseline visit to the 8-week visit, confirming the persistence of the initial PPMs in the placebo group and the false negative results of the sputum culture at visit 2 (table 2). PCR testing in culture-negative sputum samples in moxifloxacin-treated patients at 2 weeks was negative in all cases (fig. 1).

TABLE 1 Characteristics of the patients studied at randomisation

Characteristics	Placebo	Moxifloxacin
Subjects n	20	20
Age yrs	69±10	69±7
Males	19 (95)	19 (95)
Current smokers	1 (5)	1 (5)
Tobacco consumption pack-yrs	43±21	49±24
Chronic bronchitis	19 (95)	20 (100)
Cardiovascular comorbidity	8 (40)	6 (30)
More than two exacerbations in previous year	6 (30)	12 (60)
More than one hospitalisation in previous year	4 (20)	3 (15)
Post-bronchodilator FVC % pred	78±24	70±18
Post-bronchodilator FEV_1 % pred	53±16	47±15
Treatment with inhaled corticosteroids	16 (80)	15 (75)
Sputum characteristics		
Murray–Washington ⩾3	14 (70%)	14 (70%)
Haemophilus influenzae		
Subjects	11 (55)	8 (40)
Load $10^6 \times CFU \cdot mL^{-1}$	30 (0.5–60)	30 (1–50)
Haemophilus parainfluenzae		
Subjects	6 (30)	11 (55)
Load $10^6 \times CFU \cdot mL^{-1}$	0.7 (0.4–188)	0.6 (0.1–4.2)
Moraxella catarrhalis	2 (10)	2 (10)
Streptococcus pneumoniae	1 (5)	2 (10)
Enterobacteriaceae	1 (5)	0
Polymicrobial	1 (5)	3 (15)

Data presented as mean±SD, n (%) or median (interquartile range), unless otherwise stated. FVC: forced vital capacity; FEV_1: forced expiratory volume in 1 s; CFU: colony forming units.

TABLE 2 Clinical and microbiological evolution

	Placebo	Moxifloxacin	p-value
Subjects n	20	20	
At 2-week follow-up			
PPMs in sputum	14 (70)	5 (25)	0.01
Positive sputum culture	10 (50)	5 (25)	0.19
Positive PCR for PPM	4 (20)	0	0.09
PPM persistence	7 (35)	3 (15)	>0.25
Acquisition of new PPM	7 (35)	2 (10)	0.13
At 8-week follow-up			
Positive sputum culture[#]	16 (80)	15 (75)	>0.25
PPM persistence	5 (25)	1 (5)	0.18
Acquisition of new PPM	12 (60)	14 (70)	>0.25
PPMs at 8 weeks follow-up			
Haemophilus influenzae	10 (50)	4 (20)	0.10
Haemophilus parainfluenzae	7 (35)	9 (45)	>0.25
Pseudomonas aeruginosa	0	1 (5)	>0.25
Moraxella catarrhalis	1 (5)	1 (5)	>0.25
Polymicrobial	2 (10)	0	>0.25
At 5-month follow-up			
Patients with exacerbation	5 (25)	6 (30)	>0.25
Time to first exacerbation days	60±32	74±39	>0.25

Data are presented as n (%) or mean± SD, unless otherwise stated. PPM: potentially pathogenic micro-organism. [#]: one patient in placebo group with two different strains of *H. influenzae*, one persistent strain and one acquired at 8 weeks.

At 8 weeks after randomisation, bacterial persistence was still higher, although not significantly, in patients who received placebo than in moxifloxacin-treated patients (five (25%) out of 20 *versus* one (5%) out of 20, respectively; p=0.18). However, the frequencies of acquisition of a new PPM were high and similar in both treatment groups at 8 weeks, (placebo 60% *versus* moxifloxacin 70%; p>0.25) (table 2). Due to the high incidence of acquisition of a new PPM during the 8 weeks after randomisation, the prevalence of colonisation at this time point was similar between the two treatment arms (80% in the placebo and 75% in the moxifloxacin arm; p>0.25) (fig. 1).

The number of patients presenting an exacerbation during the 5-month follow-up was similar in the placebo (six (30%) out of 20) and the moxifloxacin groups (five (25%) out of 20; p>0.25). The time to the first exacerbation did not significantly differ between treatment arms (74±39 days *versus* 60±39 days for placebo and moxifloxacin arms, respectively; p>0.25).

On adjusting for age and post-bronchodilator FEV_1 (% pred) in the logistic regression model, neither moxifloxacin treatment nor the presence of persistent PPM in the follow-up sputum samples was significantly associated with the appearance of an exacerbation. However, the acquisition of a new PPM during follow-up showed a statistically significant relationship with having an exacerbation (OR 9.63, 95% CI 1.01–91.64) (table 3).

DISCUSSION

Our results have shown that a 5-day treatment course with moxifloxacin is effective in eradicating colonising bacteria in the airways of COPD patients. However, the eradication of bacteria is followed by the acquisition of a new colonising strain over a period of 8 weeks, in most cases, both in antibiotic-treated and untreated patients. The acquisition of a new strain is associated with an increased risk of developing an exacerbation.

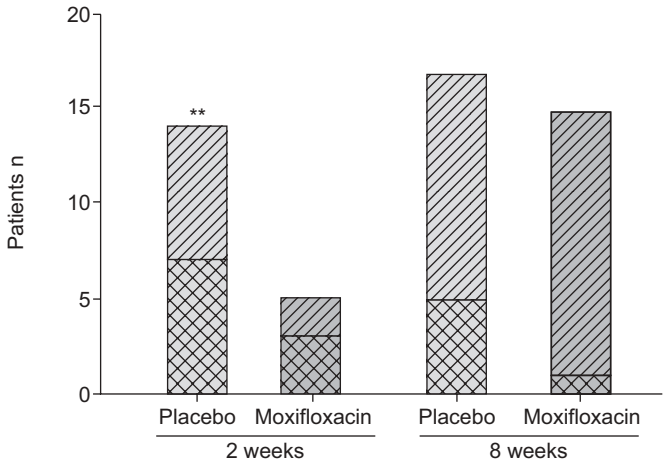

FIGURE 1. Number of patients with positive isolation of potentially pathogenic microorganisms (PPMs) in sputum at 2 and 8 weeks after randomisation. Numbers are the result of adding the percentage of patients with persistent (⊠) and newly acquired (▨) strains identified by sputum culture and detection of the DNA of PPMs by PCR (table 2). **: p=0.01.

Trials of antibiotic treatment of stable chronic bronchitis were conducted in the 1950s and 1960s, with inconclusive results [32] and, therefore, the prescription of prophylactic antibiotics for COPD has not been recommended. However, the chronic use of macrolides in COPD has received increasing attention in recent years. The main objective to the existing studies was to prevent exacerbations, either bacterial or viral, *via* mechanisms other than antibacterial activity [33–35]. In fact, two of these studies, of 3-month [34] and 1-yr duration [35], respectively, did not observe any changes in bronchial bacterial flora. However, the latter study, using erythromycin 250 mg twice daily for 1-yr, observed a significant reduction of 35% in the frequency of exacerbations compared with placebo [35]. Instead, the rationale for the use of the fluoroquinolone moxifloxacin for the treatment of stable COPD is based on its antibacterial efficacy. Bacterial eradication reduces the inflammatory burden of the

TABLE 3 Results of the logistic regression model for the variables associated with the presence of exacerbation during follow-up, adjusted for age and post-bronchodilator per cent predicted forced expiratory volume in 1 s

Variables	OR (95% CI)
Persistence of PPM isolated at randomisation	0.45 (0.04–4.51)
Treatment with moxifloxacin	1.38 (0.33–5.76)
Acquisition of a new PPM during follow-up	9.63 (1.01–91.64)

PPM: potentially pathogenic micro-organism.

airways [6, 36] and may eventually prevent the development of exacerbations [11, 14, 37, 38]. Treatment with moxifloxacin has been efficacious in eradicating bacteria from the airways but has neither prevented the occurrence of exacerbations nor prolonged the time to the first exacerbation in our population. It is important to consider that our study, although adequately powered for the main end-point, was clearly underpowered to demonstrate an effect in reduction of exacerbations or prolonging exacerbation-free interval. A previous randomised trial comparing moxifloxacin with amoxycillin, clarithromycin or cefuroxime in the treatment of exacerbations of COPD observed a significantly prolonged time free from exacerbation in favour of moxifloxacin (133 days *versus* 118 days; p=0.03) associated with a significantly better eradication rate for the quinolone [12, 14]. Interestingly, to achieve sufficient statistical power, the study included a total of 730 patients followed for 9 months after the exacerbation [14]. In contrast, an uncontrolled study in the late 1980s in patients colonised with *H. influenzae* observed persistence of 11 out of 14 strains of the microorganism after treatment with ampicillin, doxycycline, erythromycin or tetracycline, but the strains did not become resistant to the antimicrobial administered; instead, the low penetration of these antibiotics into the sputum was most likely to be responsible for the poor outcome [39].

Our results suggest that the sterilising effect of moxifloxacin in the airways vanishes after ~2 months. Therefore, to maintain the bronchial tree as free from bacteria as possible, either continuous or recurrent courses of treatment would be necessary. In this case, special attention must be paid to the possible development of resistance to the fluoroquinolone by the colonising bacteria. However, our results suggest that only exceptionally persistent strains would be repeatedly exposed to the antibiotic over time with an increased risk of development of resistance. Most of the time, newly acquired strains are exposed to the antibiotic and effectively cleared from the airways without the possibility of becoming resistant to the antibiotic. However, the long-term use of the antibiotic in this indication should be restricted to the controlled clinical trial setting, and the efficacy, safety and potential development of resistance carefully assessed.

The use of PCR and molecular typing has allowed the identification of four false-negatives for colonisation at 2 weeks among patients in the placebo group. These were very likely to be patients with a low or very low bacterial load in the airways, with sputum cultures that were negative by the usual microbiological techniques. Similarly, using the same methodology, MURPHY *et al.* [40] demonstrated that episodes of negative sputum cultures preceded and followed by identical strains of *H. influenzae* were, in fact, periods of continuous colonisation by the same strain of *H. influenzae* identified by detection of strain-specific DNA in some of the sputum samples that yielded negative cultures.

A major limitation of our study is the small sample size, which did not allow verification of the hypothesis that eradication of colonising bacteria results in a prolonged time to the first exacerbation; additionally, the number of subjects with frequent exacerbations was higher in the moxifloxacin group. It is of note that this treatment is not indicated for patients colonised with *P. aeruginosa*. Nonetheless, this colonisation occurs only in a minority of patients with COPD (only 4.2% in our screened population), and many of these episodes are transient colonisation [41]. The inclusion of *H. parainfluenzae* in the group of PPMs may be controversial; however, this microorganism was included in the original definition of PPMs [18] and has been considered a respiratory pathogen, together with *H. influenzae*, in many studies dealing with bacterial colonisation in COPD [6, 7, 9, 18, 28]. In fact, MIDDLETON *et al.* [42] demonstrated that *H. parainfluenzae* can be a pathogen in the lower respiratory tract when impaired airway defences delay bacterial clearance, as is the case in COPD. In any case, the frequency of isolation of this microorganism, both at inclusion and at 8 weeks, was similar in both treatment groups; therefore, excluding this isolation from the analysis would have not modified the results.

Of note, the study has several strengths, the randomised, double-blind design, the use of molecular typing to detect colonisation and re-colonisation by the same or different strain and, particularly, the inclusion of patients with demonstrated bacterial colonisation at baseline. Even if large, randomised, controlled trials ever demonstrate the efficacy of this therapeutic approach in reducing exacerbations and/or lung damage in COPD, this treatment will be restricted to selected subgroups of patients due to the risk of development of resistance and potential side-effects. The patients with demonstrated bacterial colonisation with sensitive bacteria are an obvious target population for this prophylactic treatment and the current study has demonstrated the efficacy of a short course of moxifloxacin in bacterial eradication in this context. Large clinical trials are required to answer some of the questions which remain open regarding antibiotic treatment of bacterial colonisation in COPD.

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STATEMENT OF INTEREST

Statements of interest for M. Miravitlles, E. Monsó, C. de la Roza, J. Morera and A. Torres, and for the study itself, can be found at www.erj.ersjournals.com/misc/statements.dtl

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DISCUSSIÓ

Prevalença i característiques de la colonització bronquial

En la present Tesi s'han investigat les característiques de la colonització bronquial en una àmplia mostra de pacients de MPOC estables ben caracteritzats mitjançant mostres d'esput espontani i induït. En estudis previs sobre colonització bronquial amb mostres obtingudes per broncoscòpia i raspall protegit entre el 25 i el 31% de les mostres presentaven cultius positius per a MPPs [12,13,18-20]. Els cultius de les mostres obtingudes per rentat broncoalveolar mostraren un percentatge similar de MPPs, d'entre el 33 i el 43% [11,18]. En aquells estudis en els quals la mostra utilitzada per identificar colonització bronquial va ser l'esput la proporció de resultats positius es situava entre el 38 i el 74% [14-17]. L'ús de la tècnica d'esput induït en aquells pacients que en període d'estabilitat no aconsegueixen expectorar facilita l'estudi de les secrecions bronquials al ampliar la mostra de pacients que poden ser inclosos, sense diferències en quant a la freqüència de colonització bronquial o el tipus d'espècies recuperades [35]. En els treballs presentats per aquesta Tesi, la prevalença de colonització bronquial trobada en esput, espontani o induït, ha estat de entre el 30% i el 75%. Aquesta troballa confirma l'alta prevalença de colonització bronquial descrita per els altres grups.

Pel que fa al tipus de microorganisme, *Haemophilus influenzae* ha estat el patogen més freqüentment aïllat (35-56%), dada que concorda amb les descrites en estudis anteriors [21,22]. Altres patògens aïllats han estat *Pseudomonas aeruginosa,* enterobacteries, *Moraxella catarrhalis, Streptococcus pneumoniae i Haemophilus parainfluenzae.*

L'anàlisi quantitativa de l'esput mesura quina és la concentració del patogen en unitats formadores de colònies. Hi ha prou evidència que la inflamació a la via

aèria augmenta amb altes concentracions de patògens colonitzadors en els pacients amb MPOC [15] i les concentracions altes també s'han associat amb una davallada de la funció pulmonar en el temps [14]. Donat que no hi ha un consens sobre quin és el punt de tall per considerar la càrrega bacteriana alta a les mostres d'esput, i en base a estudis previs sobre colonització bronquial [15,19], hem acceptat 10^5 ufc/ml com a valor per sobre del qual es considera alta càrrega bacteriana en la present Tesi Doctoral. En els nostres estudis la càrrega bacteriana va ser elevada en molts casos amb una mitjana de 10^6 ufc/ml en l'estudi 2 i de 10^4 en l'estudi 1. En l'estudi 3 el 60% dels MPPs aïllats comptabilitzaven càrregues bacterianes $>10^5$. Aquestes càrregues bacterianes altes es van produir majoritàriament quan *Haemophilus influenzae* era el germen colonitzador.

En l'estudi 1, en el qual s'analitzava l'evolució de la colonització bronquial en el temps en pacients amb MPOC moderada i sense ingressos hospitalaris per exacerbació previs, el 53% de les mostres durant el seguiment presentaven MMPs. Amb la finalitat d'investigar si aquesta colonització era deguda a la adquisició d'un nou bacteri o bé per el contrari era deguda a persistència de la mateixa soca, es van aplicar tècniques de tipatge molecular. D'acord amb aquesta anàlisi es va veure que en la major part dels casos, 30%, la colonització bronquial era deguda a l'adquisició d'una nova soca bacteriana, més que a la persistència de la mateixa soca. La persistència del mateix bacteri es va observar en el 15% de les mostres, i era bàsicament deguda a la colonització persistent per *Pseudomonas aeruginosa* i enterobactèries. Dos estudis previs [14,25] van reportar xifres de colonització bronquial persistent en el 50% dels casos, una prevalença major que la reportada en el nostre estudi. En aquests estudis, però, la major part dels pacients investigats presentaven una MPOC més greu, i només un d'ells va utilitzar tècniques de tipatge molecular per diferenciar entre una nova soca o la mateixa [25]. Les nostres troballes suggereixen que malgrat una alta prevalença de colonització bronquial en els pacients amb MPOC

moderada, la persistència del mateix microorganisme és menys freqüent, probablement degut a més recanvi en la colonització quan la malaltia està menys avançada.

Colonització bronquial i inflamació pulmonar

Recompte cel·lular

A diferència del que succeeix en l'asma, la inflamació de la via aèria observada en la MPOC es caracteritza principalment per un patró neutrofílic d'una magnitud que augmenta a mesura que empitjora la gravetat de l'obstrucció. Així es descriu, entre d'altres a l'estudi conduit per Soler i col·laboradors [18] en el qual es compara la inflamació bronquial observada en pacients amb MPOC i la mesurada en controls fumadors i no fumadors. A més a més, s'ha demostrat una associació entre colonització bronquial i la magnitud de la neutrofília, en estudis que han utilitzat el rentat broncoalveolar per obtenir les mostres de secrecions de la via aèria inferior [11,18], suggerint que la colonització bronquial pot representar un estímul independent per a incrementar la inflamació bronquial.

En l'estudi 1, la presència a l'esput de *Haemophilus influenzae, Pseudomonas aeruginosa* i enterobacteries es va associar amb una resposta neutrofílica a les secrecions bronquials, com ja prèviament havien descrit al seu treball Soler [18] i Sethi [11]. A més a més, en el període de seguiment, la persistència de colonització bronquial, present en més del 50% dels pacients, també s'associava a una resposta neutrofílica. Aquestes troballes suggereixen que els efectes de la colonització bronquial en la resposta inflamatòria en els pacients amb MPOC moderada depèn principalment de la presència d'aquests microorganismes, i suporten una relació precoç entre colonització bronquial i l'aparició de mediadors d'inflamació en les secrecions bronquials anys abans

que la malaltia produeixi danys greus. La importància de la resposta inflamatòria neutrofílica associada a la colonització bronquial es veu reforçada per la troballa d'una associació estadísticament significativa entre l'aparició d'aquesta resposta inflamatòria i una davallada de la funció pulmonar durant el seguiment. Una davallada similar en la funció pulmonar associada a colonització bronquial havia estat documentada per Wikilson i col·laboradors [14], però no per Hill i col·laboradors [15]. En el nostre estudi la davallada del VEMS en els pacients MPOC amb neutrofília a l'esput en alguns casos s'aproxima a la observada en pacients MPOC més greus i amb exacerbació. Aquesta troballa avala la hipòtesi de que la colonització bronquial té un impacte subclínic en els pacients amb MPOC moderada en absència de símptomes d'exacerbació, a diferència dels pacients més greus, que identifiquen els empitjoraments en la dispnea fàcilment, inclús quan els canvis en la funció pulmonar són petits.

En l'estudi 2, el recompte cel·lular no mostrava diferències significatives entre els pacients colonitzats i els no colonitzats, i tots dos grups presentaven una neutrofília superior al 65%, valor considerat com el límit alt de la normalitat [36,37], un patró inflamatori neutrofílic similar al descrit per Barnajee i col·laboradors [17] en el qual les mostres d'esput presenten neutrofília, amb un augment no significatiu en el grup de pacients colonitzats. En aquest estudi els pacients presentaven un VEMS inferior a l'observat en l'estudi 1, a l' igual que en l'estudi de Barnajee, dada que molt probablement explica la manca d'associació entre neutrofília i la presència de MPPs a l'esput, per la minimització del efecte d'aquesta colonització front a la magnitud de la inflamació local pròpia de la malaltia avançada.

Mediadors d'inflamació

En els estudis 1 i 2, la presència de MPPs a l'esput s'associava amb un increment de interleuquines a les secrecions bronquials, tal i com ja havia estat descrit prèviament [11,15,17,18,24]. En l'estudi 2, la colonització bronquial es va

associar significativament amb un augment dels nivells d'IL-1β, IL-6 i la IL-8 en esput. A més a més, quan el patogen colonitzador era *Haemophilus influenzae* els nivells de IL-8 i de IL-1β eren significativament majors respecte els valors observats quan altres patògens eren els colonitzadors, amb una relació dosis-resposta entre la càrrega bacteriana i els nivells de IL-8 que justifica la hipòtesi etiològica. La IL-8 és un conegut quimio-atraient i activador de neutròfils [37], i la relació entre la colonització bronquial i el nivell d'aquesta citocina en el sobrenedant d'esput ha estat prèviament reportada tant en el model cel·lular [38], com en els pacients amb MPOC [39,40], amb una relació dosi-resposta amb la càrrega bacteriana en diversos estudis [15,17]. IL-1 és una família de citocines que comprèn 11 proteïnes que han demostrat un paper central en diverses malalties inflamatòries [41].

En l'estudi 1, la presència de *Haemophilus influenzae* en el seguiment estava significativament associada a neutrofília i a concentracions elevades a l'esput de IL-1β i IL-12, amb una relació dosi-depenent entre la càrrega bacteriana, la neutrofília i la IL-1β. Ja en altres estudis no focalitzats en *Haemophilus influenzae* havia estat descrita la relació dosi-resposta entre la càrrega bacteriana de MPPs i inflamació bronquial a la MPOC en estabilitat, en la qual a major càrrega bacteriana majors concentracions dels marcadors d'inflamació, observacions que han recolzat el paper important de la colonització bronquial en la patogènesis de la inflamació en la MPOC [14,15,18,39].

En l'estudi 1, la resposta inflamatòria associada a la presència en esput de MPPs no es va observar quan el microorganisme aïllat era *Haemophilus parainfluenzae*, fet que suggereix que l'efecte d'aquest bacteri sobre la resposta inflamatòria a mucosa bronquial ha de ser considerat marginal. Aquesta hipòtesi es veu reforçada per altres estudis en els quals *Haemophilus parainfluenzae* ha mostrat en cultius cel·lulars una baixa adherència a la mucosa bronquial [48] i en observacions en pacients amb bronquitis crònica, on

el bacteri ha evidenciat una baixa capacitat d'induir una resposta inflamatòria bronquial [49]. Així, cal considerar *Haemophilus parainfluenzae* com un bacteri amb baixa capacitat d'induir inflamació a la mucosa bronquial, i per aquest motiu en l'estudi 2 es va optar per no incloure aquest microorganisme entre els patògens considerats.

En l'estudi 3 no es va trobar un increment de les concentracions de citocines a l'esput en els pacients amb colonització bronquial. Diferents raons expliquen aquet fet, essent les més importants la presència de càrregues bacterianes baixes, i el fet de que en aquest estudi hi hagués molts aïllaments de *Haemophilus parainfluenzae*, (30%), amb un menor impacte sobre la inflamació bronquial, segons s'ha comentat prèviament. Quan en el estudi es van categoritzar els pacients d'acord amb càrrega bacteriana alta ($>10^5$) o baixa, es van observar majors concentracions de citoquines a l'esput dels pacients amb càrregues més elevades, que no va arribar a la significació molt probablement per la grandària de la mostra i la variabilitat observada en la mesura.

Colonització bronquial i Inflamació sistèmica

És ben conegut que durant les aguditzacions de MPOC augmenten els nivells de marcadors d'inflamació sistèmica com la proteïna-C-reactiva [44,45] i el fibrinogen en sèrum [46]. També és àmpliament acceptat que els pacients de MPOC presenten un patró d'inflamació sistèmica durant els períodes d'estabilitat. Gan i col·laboradors [47] en una metaanàlisi van reportar elevacions significatives de proteïna-C-reactiva en pacients de MPOC en fase d'estabilitat clínica, comparats amb individus sans. En un segon treball aquests nivells elevats de proteïna-C-reactiva van ser predictors d'hospitalització per MPOC i mort [48]. Man i col·laboradors [49] han estudiat la cohort de MPOC inclosa en el "Lung Health Study", i han observat que els pacients que

presentaven valors de proteïna-C-reactiva per sobre de 7.06 mg/l tenien més risc de patir accidents cardiovasculars i mort que els pacients MPOC amb baixes concentracions de proteïna-C-reactiva. En el nostre estudi els nivells de proteïna-C-reactiva trobats en els pacients no colonitzats (3.5 [1.7-5.4] mg/l), ha estat similar als nivells descrits en pacients de MPOC en fase estable [45]. Nivells molt més elevats de proteïna-C-reactiva (6.5 [2.5-8.5] mg/l), però, van ser observats en els pacients colonitzats del nostre estudi, un terç dels quals superaven el valor de 7.06 mg/l. La associació mantenia la significació estadística després d'ajustar per covariables (OR ajustada 2.57, 95% IC 1.07-6.18), sent el VEMS l'única variable amb una relació inversament proporcional i estadísticament significativa amb els nivells de proteïna-C-reactiva.

Aquestes troballes recolzen la hipòtesi d'un efecte directe de la colonització bronquial sobre la inflamació sistèmica. Així, la colonització bronquial pot suposar un factor de risc per a inflamació sistèmica identificable pels nivells de proteïna-C-reactiva, indetectable si no es realitzen cultius d'esput en estabilitat.

Efecte de la colonització bronquial espècie-específica per *H. influenzae*

La colonització bronquial és un procés dinàmic amb canvis en el tipus de patogen colonitzador, canvis en les soques d'un mateix bacteri i en la seva càrrega bacteriana al llarg del temps [14,25]. Les diferents espècies bacterianes i les diferents soques de la mateixa espècie difereixen en virulència i capacitat de produir inflamació [9,15]. En l'estudi de Hill i col·laboradors *Pseudomonas aeruginosa* va ser el bacteri que va demostrar més potencia inflamatòria seguit de *Haemophilus influenzae*, al contrari que *Moraxella catarrhalis* que va mostrar menys capacitat inflamatòria [15]. En un altre estudi, Sethi i col·laboradors van trobar que els patògens que produïen mes inflamació eren *Haemophilus influenzae* i *Moraxella catarrhalis* [9]. En els nostres treballs *Haemophilus*

influenzae ha estat el patogen colonitzant més prevalent, tal i com està descrit en treballs previs, i amb la major càrrega bacteriana. Analitzant els efectes espècie específics que la colonització per aquest microorganisme pot produir en els pacients de MPOC en fase estable hem objectivat en l'estudi 1 que la presència de *Haemophilus influenzae* es relacionava amb una resposta inflamatòria a la mucosa bronquial caracteritzada per una inflamació neutrofílica i altes concentracions de IL-1β i IL-12 a l'esput. En l'estudi 2, la presència d'aquest bacteri s'associava a un major consum de tabac acumulat, després d'ajustar per variables com el tabaquisme actual i el grau d'obstrucció bronquial. També s'associava a una clara inflamació bronquial, posada de manifest pels elevats nivells de IL-1β I IL-8. A més a més, la càrrega bacteriana i els nivells de IL-8 mostraven una relació dosi-resposta. Pel que fa també a les manifestacions sistèmiques associades a la colonització, en els pacients colonitzats per *Haemophilus influenzae* es va detectar un empitjorament de la qualitat de vida mesurada per Qüestionari de St.George en els dominis d'activitat i impacte, no presents de manera significativa quan els pacients es colonitzen per altres microorganismes. Aquests resultats són consistents amb les troballes del treball de Barnajee i col·laboradors [17] a on es va trobar un empitjorament de la qualitat de vida en MPOC amb colonització bronquial, en la majoria de cultius positiva per *Haemophilus influenzae.*

Factors de risc per a colonització bronquial

En l'estudi 3, la colonització bronquial s'associava al consum acumulat de tabac, al grau de dispnea, a les comorbiditats i a la història d'exacerbacions i hospitalitzacions l'any previ. En altres estudis, el tabaquisme actual i la obstrucció bronquial greu han estat identificats com a factors que predisposen a patir colonització bronquial [20,29]. En els nostres estudis, però, no hem observat diferències en la funció pulmonar entre colonitzats i no colonitzats.

Així, en base al nostre estudi cal considerar la relació entre funció pulmonar i colonització bronquial com a poc consistent, en la línia d'altres estudis que tampoc han pogut demostrar aquesta associació [11,15,18]. Això pot ser en part atribuïble, almenys en part, a la baixa representació de pacients amb MPOC moderada a la majoria de sèries clíniques, cosa que també succeeix en el nostre estudi. Feta aquesta precisió, és interessant el fet que els únics dos factors identificats a l'anàlisi multivariat que estan independent i significativament associats amb la presència de MPPs a l'esput en el nostre estudi han estat l'alta percepció de dispnea, a més del color de l'esput, ja que el grau de dispnea és un marcador de severitat de la malaltia, que engloba components addicionals a la funció respiratòria a la MPOC.

Marcadors de colonització bronquial

Per facilitar el diagnòstic de colonització bronquial, l'ús de marcadors substituts pot ser interesant. En l'estudi 3, la purulència de l'esput, és a dir, el seu color, graduat per una simple escala de 1 a 5, ha revelat diferències significatives de color entre pacients colonitzats i no colonitzats, revelant-se com un potent indicador de la presència de cultius positius per MPPs i de càrregues bacterianes elevades. Pacients que presenten color de l'esput valorat en la escala utilitzada com a 3 o superior, que correspon a colors del groc fosc al verd, presenten una prevalença de colonització bronquial superior al 80%. La rellevància del color de l'esput ja ha estat descrita i validada per les exacerbacions de la MPOC en les quals esputs groguencs o verdosos estan significativament associats amb una etiologia bacteriana, en comparació amb els esputs blanquinosos [50,51]. La relació entre el color de l'esput i la colonització bronquial, però, ha estat fins ara poc investigada [15].

Tractament antibiòtic de la colonització bronquial

El reconeixement del potencial paper dels MPPs en el manteniment de la inflamació bronquial en el pacient amb MPOC en fase estable ha plantejat el tractament amb antibiòtics de la infecció bacteriana, a més a més dels períodes d'exacerbació, en els períodes d'estabilitat. Els nous antibiòtics, més actius, podrien permetre tandes curtes de tractament administrades amb la intenció d'eradicar els bacteris de la via aèria. En aquest context, hem portat a terme l'estudi 4, en el qual s'ha comparat l'efectivitat de moxifloxací front a placebo en la eradicació de la colonització bacteriana, mitjançant una assaig clínic amb grup control, aleatoritzat i doble cec.

Els primers assajos clínics amb antibiòtics en la MPOC estable es van dur a terme a la dècada dels anys 50 i 60 [30], amb resultats poc concloents, per la qual cosa la prescripció d'antibiòtic de manera profilàctica per la MPOC no es va considerar recomanable en aquell moment. L'ús crònic de macròlids en la MPOC ha despertat de nou interès es els últims anys, però, amb interès per analitzar la capacitat preventiva de la exacerbació, bacteriana o vírica, per mecanismes diferents a l'activitat bactericida pròpia del macròlid [31,52,53]. De fet, en dos d'aquests estudis, un a 3 mesos [54], i un altre a 1 any de duració [31], no van observar canvis en la flora bacteriana bronquial. Aquest últim estudi, però, emprant eritromicina 250 mg/12 hores durant 1 any, va observar una reducció significativa del 35% en la freqüència de les exacerbacions en comparació amb placebo [31].

Contràriament a la utilització de macròlids, l'ús de fluorquinolones pel tractament de la MPOC estable es basa en la activitat antibacteriana del fàrmac. Moxifloxací és una fluorquinolona de quarta generació que ha demostrat activitat bactericida en front dels patògens més freqüentment aïllats a les secrecions respiratòries dels pacients amb MPOC [55,56] i eficàcia clínica en el

tractament de les exacerbacions [57,58]. L'eradicació bacteriana redueix la càrrega inflamatòria de la via aèria [15,59], i per aquesta via prevé les aguditzacions [13,57,60,61].

Els resultats del estudi realitzat han mostrat que un tractament de 5 dies amb moxifloxací és eficaç per eradicar les bactèries colonitzadores de la via aèria dels pacients amb MPOC en fase estable. Aquesta eradicació, però, és seguida en molts casos per l'adquisició d'una nova bactèria en un període d'unes 8 setmanes en els pacients tractats. L'adquisició d'una nova bactèria es va associar amb un increment en el risc de patir una exacerbació, independentment del tractament previ. El tractament amb moxifloxací, per tant, va ser eficaç eradicant la colonització però no va ser capaç de prevenir les exacerbacions ni d'allargar el període fins a la primera exacerbació en la mostra estudiada. És important considerar que el nostre estudi, encara que suficientment potent per demostrar l'objectiu primari, no ho és per demostrar un efecte en la reducció d'exacerbacions o en la prolongació del període lliure d'exacerbacions. Un assaig clínic randomitzat previ comparant moxifloxací amb amoxicil·lina, claritromicina i cefuroxima en el tractament de les exacerbacions de la MPOC ha observat una prolongació significativa del temps lliure d'aguditzacions a favor de moxifloxací (133 dies *versus* 118 dies; p=0.03), i una raó d'eradicació millor per la quinolona [55,57]. En aquest estudi, però, per aconseguir suficient poder estadístic es van haver d' incloure 730 pacients, els quals es va seguir durant 9 mesos després de l'exacerbació.

Els nostres resultats suggereixen que l'efecte esterilitzant del moxifloxací desapareix després d'aproximadament 2 mesos. Es per això que per mantenir l'arbre bronquial lliure de bacteris semblaria necessària l'administració mantinguda d'antibiòtic o en tandes recurrents amb freqüència no superior a aquest temps. En aquesta aproximació, però, cal fer especial atenció a l'aparició de resistències a les quinolones en els bacteris colonitzadors. Els nostres

resultats, però, suggereixen que només de manera excepcional bactèries persistents es veurien sotmeses a l'exposició repetida de l'antibiòtic amb el consegüent risc de desenvolupar residències. Durant la majoria del temps de tractament serien noves bactèries les que es veuen exposades a l'antibiòtic, successivament eradicades eliminant la possibilitat de resistència. Malgrat això,, l'ús perllongat d'antibiòtics amb aquesta indicació s'ha de limitar als assajos clínics per valorar l'eficàcia, la seguretat i el desenvolupament de resistències, fins que no hi hagi més evidencies que suportin la utilització d'aquesta teràpia preventiva.

En el nostre estudi, l'ús de la reacció en cadena de la polimerasa i del tipatge molecular, ha permès la identificació de 4 falsos negatius per colonització a les 2 setmanes en el grup de placebo. Probablement aquests pacients tenien càrregues bacterianes baixes, motiu pel qual els cultius microbiològics d'esput convencionals van ser negatius. D'una manera similar, Murphy i col·laboradors ven demostrar que episodis de cultius d'esput negatius precedits i seguits per soques idèntiques de *Haemophilus influenzae* eren, de fet, períodes de colonització persistent per la mateixa soca [62].

Una limitació important en el nostre estudi és el baix nombre de pacients participants, que no permet verificar la hipòtesi que l'eradicació bacteriana prolonga el temps fins a la propera agudització. A més a més, el nombre de pacients amb freqüents aguditzacions l'any previ
va ser més gran en el grup tractat amb moxifloxací. El nostre estudi, però, posseeix moltes fortaleses com el ser randomitzat i doble cec, l'ús de tipatge molecular que permet detectar colonització i re-colonització per una mateixa soca bacteriana i la inclusió només de pacients amb colonització bronquial demostrada en el moment basal. Els pacients amb colonització bronquial per un bacteri sensible són òbviament la població diana pel tractament profilàctic, i el nostre estudi ha demostrat l'eficàcia de una tanda curta de moxifloxací en

l'eradicació bacteriana en aquest context. Es requereixen estudis més grans per respondre a les qüestions que queden encara pendents per justificar el tractament antibiòtic de la colonització bronquial en la MPOC. Tot i que assajos clínics aleatoritzats amb més pacients demostressin l'eficàcia d'aquesta aproximació terapèutica en la reducció d'exacerbacions, aquest tractament caldria que fos restringit a grups seleccionat de pacients, pel risc de desenvolupar resistències i d'efectes secundaris no desitjats.

CONCLUSIONS

L'arbre bronquial dels pacients amb MPOC moderada en període d'estabilitat clínica està freqüentment colonitzat per MMPs, principalment per *Haemophilus influenzae.*

La presència de MPPs en les secrecions bronquials en període d'estabilitat clínica dels pacients de MPOC s'associa a un augment en els nivells de marcadors d'inflamació bronquial i també sistèmica, com ara l'augment de la proteïna-C-reactiva, d'una magnitud suficient per produir efectes significatius en el curs de la malaltia.

La colonització bronquial per *Haemophilus influenzae* produeix un patró inflamatori bronquial espècie-específic que s'associa amb un empitjorament en la qualitat de vida dels pacients de MPOC. Aquestes troballes suggereixen que l'impacte de la colonització bronquial per *Haemophilus influenzae* pot superar el d'altres patògens colonitzants.

L'impacte de la colonització bronquial per *Haemophilus parainfluenzae* es demostra inferior al de la resta de MMPs i per aquest motiu el seu aïllament a les secrecions bronquials s'hauria de considerar dins del grup dels gèrmens no patògens.

La colonització bronquial s'associa a un major consum acumulat de tabac, a una major dispnea, i a canvis en la coloració de l'esput. Aquest últim, pot representar un marcador de la presència de colonització bacteriana senzill d'utilitzar.

L'ús de moxifloxací en una tanda curta ha demostrat ser eficaç en l'eradicació de la colonització bronquial en els pacients amb MPOC. L'efecte d'aquesta eradicació, però, es perd en un període de dos mesos. Es requereixen assajos

clínics amb més pacients per verificar l'eficàcia i seguretat d'aquesta pràctica terapèutica així com la freqüència d'aparició de resistències.

LIMITACIONS DELS ESTUDIS

En l'estudi 1 i 2, no hem realitzat anàlisi genòmic de les mostres d'esput en les quals no creixien MPPs, i no podem descartar infradiagnòstic de colonització bronquial amb càrregues per sota del límit de detecció del cultiu d'esput. Aquesta colonització ha de considerar-se com inusual, però, perquè quan aquest enfocament diagnòstic s'ha utilitzat, s'ha identificat colonització bronquial només en una dècima part dels cultius d'esput negatius [63].

En l'estudi 2, la selecció dels pacients inclosos en l'estudi PAC-EPOC posa límits en l'extrapolació dels resultats, que no es poden aplicar a pacients greus, perquè els participants en aquest estudi havien d'haver estat hospitalitzats només un cop per a una exacerbació de la MPOC en base als criteris de selecció utilitzats. L'enfocament seguit en l'estudi, però, determina que la colonització bronquial, la variable principal considerada en el present estudi, no hagués estat modificada pels ingressos recurrents, que introdueixen a gèrmens propis de l'hospital en la flora colonitzadora. Per tant, aquesta limitació aparent, de fet, augmenta la força de les conclusions, que s'apliquen clarament als pacients MPOC i baixa freqüentació hospitalària.

Els resultats de l'estudi 3 s'han d'interpretar considerant que no ha estat possible la determinació de la concentració dels marcadors inflamatoris a l'esput en totes les mostres, en la majoria dels casos a causa d'una escassa recuperació d'esput que no proporcionava suficient sobrenedant per a la quantificació dels mediadors de la inflamació. El disseny transversal de l'estudi tampoc va permetre examinar la dinàmica i l'evolució en el temps de la colonització bacteriana i la inflamació de les vies aèries durant les exacerbacions.

En l'estudi 4, una limitació important és el baix nombre de pacients participants, que no permetia verificar la hipòtesi que l'eradicació bacteriana era capaç de perllongar el temps fins a la propera agudització.

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