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Mise au point de l'analyse par séquençage à haut-débit du microbiote fongique et bactérien respiratoire chez les patients atteints de mucoviscidose

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ABREVIATION

ABPA	Allergic bronchopulmonary aspergillosis (Aspergillose broncho-pulmonaire allergique)
ABPM	Allergic bronchopulmonary mycosis
ADNr 16S	ADN ribosomal 16S
ANOSIM	Analysis of similarity
BAL	Broncho-alveolar lavage
BDPEE	Biologie et Diversité des Pathogènes Eucaryotes Emergents
BP	Brossages bronchiques protégés
BPCO	Broncho-pneumopathie chronique obstructive
CF	Cystic fibrosis
CFF	Cystic fibrosis foundation
CFTR	Cystic fibrosis transmembrane regulator
CFU	Clony-forming unit
COPD	Chronic obstructive pulmonary disease
CRCM	Centres de ressources et de compétences de la mucoviscidose
CRD	Chronic respiratory diseases
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
ECMM	European confederation for medical mycology
FEV1	Forced expiration volume at the end of the first second
F508del/ΔF508	Mutation delta F508 responsable de perte de phénylalanine (F) en position 508 de la protéine CFTR
HMP	Human microbiome project
HTS	High-throughput sequencing
IA	Invasive aspergillosis
Ig	Immunoglobulin
IL	Interleukin
IFNγ	Interferon gamma
IPA	Invasive pulmonary aspergillosis
ISHAM	International society for human and animal mycology
ITS	Internal transcribed spacer
LBA	Lavage broncho-alvéolaire

MetaHIT	Metagenomics of the human intestinal tract consortium
MFIP	MucoFong international project
NGS	Next generation sequencing (séquençage à haut-débit)
NIH	National institutes of health
OTU	Operational taxonomic unit
ORL	Oto-rhino-laryngologie
OW	Oropharyngeal wash
PCA	Principal componant analysis (Analyse principale composante)
PMA	Propidium monoazide
q-PCR	PCR quantitative ou PCR en temps réel
RNA	Ribonucleic acid
VEMS1	Volume expiratoire maximal pendant la première Seconde

LEXIQUE

ADNr 16S : Gène codant pour la sous-unité 16S des ribosomes bactériens qui est hautement conservée entre les différentes espèces bactériennes. Le séquençage du gène de l'ARNr 16S, en particulier les régions qui sont variables parmi les espèces nous permet de classifier les bactéries jusqu'au niveau des espèces.

ANOSIM (Analysis of similarity) : un test statistique comparant la différence entre deux groupes

Diversité Alpha : La diversité mesurée à l'intérieur d'un échantillon, qui estime la richesse et « l'evenness » à l'intérieur d'un échantillon, (par exemple : indice de Shannon, indice de Simpson, CHAO1, cf. Annexe 5).

Diversité Beta : Mesure de la divergence de la diversité entre les échantillons, qui est souvent utilisée pour montrer la différence de diversité associée à des conditions différentes d'un écosystème à plusieurs temps ou entre plusieurs sites. Elle représente la similitude de composition et diversité bactérienne entre les échantillons (par exemple indice de Brays-Curtis).

« Evenness » : Les nombres relatifs des unités taxonomiques dans un échantillon.

HMP : Le projet américain « Human microbiome project » (http://www.hmpdacc.org/), financé par le « National Institutes of Health » pour caractériser la composition de l'ensemble du microbiome humain (son abondance et sa diversité) et son rôle dans la santé humaine et les maladies.

ITS (Internal Transcribed Spacer): c'est une région de l'ADN ribosomique fongique, incluant la région ITS1 qui se trouve entre les gènes 18S et 5,8S et la région ITS2 qui se trouve entre les gènes 5,8S et 28S. Ce sont des régions non codantes, hautement polymorphes et multicopies dans les génomes, qui sont entourées par des régions hautement conservées permettant l'emploi d'amorces universelles afin de déterminer les séquences de régions variables adjacentes amplifiées par PCR. Pour ces raisons, ils sont très fréquemment utilisés dans le séquençage à haut-débit pour permettre d'identifier en taxonomie les microymycètes.

Librairie : Préparation d'un fragment d'ADN cible, dans notre étude : les régions V3-V5 d'ADNr 16S ; le locus ITS2, et ligation aux index Ionexpress barcoding à l'issue d'une PCR en point final.

Métagénome : La collection de tous les gènes obtenus à partir des microorganismes dans un habitat donné.

Microbiome : le terme « biome » (bactériome, mycobiome, virome) désigne l'ensemble des génomes des microorganismes (bactéries ou micromycètes ou virus et phages) composant une communauté microbienne et qui sont soumis à des pressions environnementales du site.

Microbiote : l'ensemble des microorganismes résidant dans un site (un tissu ou organe donné).

NGS (Next generation sequencing) : La nouvelle génération de séquençage, technologie à haut-débit, qui permet un séquençage rapide d'ADN multiparallèlisé (par exemple les plateformes Roche 454 Pyroséquençage, IonTorrent, Illumina HiSeq, Illumina MiSeq, …).

OTU (Operational Taxonomic Unit) : Catégorisation des organismes en espèces ou groupes d'espèces à partir des données de séquençage à haut-débit selon des critères de similitude moléculaire.

PCA (pour Principal component analysis) : L'analyse en composante principale est une méthode multidimensionnelle/factorielle permettant d'étudier la structure des liaisons linéaires de l'ensemble des variables considérées et de détecter les facteurs les plus corrélés. Dans notre étude, la PCA a permis d'estimer la similitude et les changements observés dans la composition microbienne entre les groupes d'échantillons.

Pipeline : Terme utilisé en informatique correspondant à l'enchaînement coordonné d'étapes informatiques élémentaires.

Reads : séquences d'ADN obtenues après la réalisation du séquençage à haut-débit.

Richesse : Nombre d'unités taxonomiques uniques détectées dans un échantillon.

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I. RESUME

L'infection broncho-pulmonaire représente le problème majeur des malades atteints de la mucoviscidose. Plusieurs bactéries sont connues depuis des dizaines années comme les principaux agents responsables de ces infections (par exemple *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia*, *Achromobacter xylosoxidans…*). Récemment, certains genres fongiques notamment les champignons filamenteux (comme *Aspergillus*, *Scedosporium*…) ont été identifiés comme des pathogènes émergeants ou ré-émergeants pouvant être responsables d'infection invasive. Ainsi, la détection des microorganismes impliqués dans ces colonisations et/ou infections respiratoires demeure importante sur le plan physiopathologique et clinique.

Si la culture microbiologique reste la méthode la plus utilisée à ce jour pour le diagnostic des infections microbiennes, elle ne permet pas d'identifier les microbes non-cultivables ou difficiles à cultiver. Depuis quelques années, grâce au développement de la technique moléculaire de séquençage à haut-débit (next generation sequencing ou NGS), plusieurs études ont montré que l'écologie microbienne du poumon des patients atteints de la mucoviscidose est très complexe et correspond à une flore poly-microbienne, appelée le microbiote pulmonaire, comprenant non seulement des bactéries mais également des micromycètes (levures et/ou champignons filamenteux) et des virus et phages. Une dysbiose (modification en abondance et diversité) de cette flore pourrait influencer la fonction respiratoire et l'état clinique du patient.

Alors que le microbiome bactérien et son rôle en pathogenèse sont largement étudiés, peu d'études ont porté sur la composante fongique (mycobiote/mycobiome) du microbiote pulmonaire. Notre travail de thèse s'inscrit dans les différents projets développés au sein de l'axe de recherche « Microbiote pro- et eucaryote pulmonaire » coordonné par le Pr Laurence Delhaes dans l'équipe Biologie et Diversité des Pathogènes Eucaryotes Emergeants (BDPEE) dirigée par le Dr Eric Viscogliosi. Il se focalise sur l'analyse NGS du microbiote pro- et eucaryotique respiratoire chez les patients atteints de la mucoviscidose et notamment la comparaison de différentes approches méthodologiques en vue d'une optimisation et standardisation de la méthode.

Dans un premier temps, nous présenterons une synthèse des connaissances actuelles d'une part des phénomènes de colonisations/infections fongiques chez les patients atteints de mucoviscidose et d'autre part dans le domaine du microbiote pulmonaire et surtout du mycobiote pulmonaire autour duquel notre équipe se focalise.

Dans un deuxième temps, nous avons travaillé à mieux adapter l'approche NGS aux études du microbiote pulmonaire dans la mucoviscidose. En effet, le séquençage à haut-débit est une technique puissante mais pour laquelle des biais peuvent être introduits à de nombreuses étapes méthodologiques. Un des biais les plus importants est que l'approche NGS ne permet pas de différencier les microorganismes vivants, des cellules mortes ou endommagées, ni de l'ADN extracellulaire. Dans le contexte de notre travail –celui du microbiote pulmonaire chez des patients atteints de mucoviscidose et souvent exposés aux antibiotiques par voie intraveineuse à forte dose, l'analyse NGS pourrait évaluer incorrectement l'abondance et la diversité de ce microbiote pulmonaire. Un prétraitement des échantillons par propidium monoazide (PMA), qui permet de cibler sélectivement l'ADN des cellules vivantes, pourrait être une solution pour palier à cette limite. Notre étude avait donc comme objectif de déterminer si un prétraitement par PMA des expectorations modifiait le microbiote pro- et eucaryote pulmonaire analysé par NGS. Nous discutons l'intérêt et la relevance clinique de cette approche « PMA - NGS » permettant une quantification isolée des microorganismes vivants dans le contexte de la mucoviscidose.

II. ABSTRACT

Chronic pulmonary infection results in an irreversible decline in lung function in patients with cystic fibrosis (CF). While several bacteria are known as main causes for these infections (for example: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia cepacia*, *Achromobacter xylosoxidans*...), more recently some fungal genera including filamentous fungi (such as *Aspergillus*, *Scedosporium*...) have also been identified as emerging or re-emerging pathogens able to cause invasive mycosis. Thus, the identification of the microorganisms involved in the respiratory colonizations and/or infections has become essential.

Still now culture methods remain the gold standard for diagnostic of microbial infections. However, it could not identify non-culturable or difficult-to-cultivate microorganisms. Thanks to the development of high-throughput sequencing (next generation sequencing or NGS), recent studies have shown that the lung of patients with CF is a complex poly-microbial flora, also called the CF lung microbiota, which includes not only bacteria but also fungi (yeast and/or filamentous fungi), and viruses and phages. Dysbiosis (loss of abundance and/or diversity) of the lung microbiota has been associated with the patient's decreased lung function and poor clinical status.

While lung bacteriota and its role in pathogenesis have widely been studied, few research studies focus on the fungal component (mycobiota/ mycobiome) of the lungs. Our thesis (PhD work) focuses on NGS analysis of pro- and eukaryotic lung microbiota in CF patients, in particular on the comparison of different methodological approaches to optimize and standardize the NGS protocol. This project has been developed under the supervision of Pr. Laurence Delhaes in the “Biology and Diversity of Eukaryotic Emerging Pathogens” team directed by Dr. Eric Viscogliosi.

Firstly, we present a state of art on the current knowledge on the fungal colonization/infections risk in CF as well as the development of new concepts of lung microbiota and lung mycobiota on which our team focuses.

Secondly, we applied the NGS approach to study the pro- and eukaryotic microbiota in the sputum samples of CF patient lung. Indeed, NGS is a powerful technique that may introduce biases on numerous methodological steps. One of the most important biases is that this technique could not differentiate among the living microorganisms, the dead or damaged cells, and the extracellular DNA. In the context of the CF lung microbiota which is often exposed to high-dose intravenous antibiotics, the analysis by NGS might evaluate

inaccurately the abundance and the diversity of the lung microbiota. Pretreatment of samples by propidium monoazide (PMA), which can target selectively the DNA of viable cells, could be a solution to overcome this limitation. Our study aimed to determine whether a sample pretreatment with PMA modified the lung pro- and eukaryotic microbiota analyzed by NGS. We discuss the clinical relevance of this approach "PMA - NGS" in the context of CF patients to a better quantification of living microorganisms.

III. INTRODUCTION GENERALE ET OBJECTIF

L'infection broncho-pulmonaire représente le problème majeur chez les malades atteints de mucoviscidose. Cette infection est généralement chronique, causée par des colonisations et/ou infections microbiennes, une réaction inflammatoire inhérente, et des phénomènes d'exacerbations aigües intercurrentes détériorant progressivement la fonction respiratoire, ce qui conditionne en grande partie le pronostic vital des patients (Ratjen and McColley, 2012).

Aujourd'hui, le diagnostic et le suivi de ces colonisations/infections sont encore réalisés essentiellement par les méthodes conventionnelles (examens directs et cultures microbiologiques). Cette approche permet de détecter des microorganismes vivants ayant un rôle important dans l'évolution et/ou la physiopathologie de la mucoviscidose. En utilisant ces méthodes culture-dépendantes, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia*, *Achromobacter xylosoxidans*, *Stenotrophomonas maltophilia* ont été identifiés comme les principaux agents bactériens responsables des infections respiratoires dans la mucoviscidose dont les infections pulmonaires chroniques à *P. aeruginosa* sont une cause majeure de mortalité chez les patients adultes (Ciofu *et al.*, 2013). Récemment, certains genres fongiques notamment les champignons filamenteux (comme *Aspergillus*, *Scedosporium,…*) ont été identifiés comme des pathogènes émergents ou ré-émergents pouvant être responsables d'infections invasives (Touati *et al.*, 2014). Depuis quelques années, les études montrent que l'écologie microbienne du poumon des patients atteints de mucoviscidose est très complexe et correspond à une flore poly-microbienne, comprenant non seulement des bactéries mais également des micromycètes (levures et/ou champignons filamenteux) ainsi que des virus et des phages (Sibley *et al.*, 2006; Filkins and O'Toole, 2015). Il nous est donc apparu essentiel d'étudier l'ensemble de cette flore poly-microbienne et d'appréhender les interactions potentielles de ces agents microbiens de façon à améliorer notre compréhension physiopathologique de la pathologie pulmonaire mucoviscidosique ainsi que la prise en charge thérapeutique de cette maladie.

Cependant, les méthodes basées sur la culture microbiologique ne permettent pas d'identifier les microorganismes non-cultivables ou difficiles à cultiver et reflètent peu les co-infections et la dynamique des différentes populations microbiennes. Depuis le début des années 2000, l'approche culture-indépendante par séquençage à haut-débit (ou NGS) qui permet la lecture de plusieurs milliers de séquences en parallèle, génère des données moléculaires très détaillées sur un échantillon donné. Cette approche NGS a modifié notre connaissance de l'écologie microbienne humaine, appelé le microbiome humain. Dès 2010, certains

chercheurs ont pu montrer grâce au NGS que les poumons humains n'étaient pas aussi stériles que nous le considérions (Erb-Downward *et al.*, 2010; Hilty *et al.*, 2010; Charlson *et al.*, 2010). Bien que le nombre de microorganismes dans le poumon ne soit pas aussi élevé que celui du microbiome intestinal, les voies respiratoires humaines représentent un écosystème spécifique, appelé le microbiome/microbiote pulmonaire et contenant de nombreuses bactéries aérobies, anaérobies strictes, des champignons et des virus (pour revues voir (Dickson *et al.*, 2013; Marsland and Gollwitzer, 2014; Dickson *et al.*, 2015b; Nguyen *et al.*, 2015; Nguyen and Delhaes, 2015; Adar *et al.*, 2016)). Malgré des résultats variables selon les articles publiés, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* ont été identifiés comme les phyla les plus fréquents. Au niveau du genre bactérien, *Streptococcus*, *Prevotella*, *Veillonella*, *Porphyromonas*, *Fusobacteria* sont prédominants, constituant le « core taxa » du microbiote bactérien pulmonaire, avec d'autres pathogènes potentiels tels que *Haemophilus* et *Neisseria* qui sont moins représentés (Erb-Downward *et al.*, 2010; Hilty *et al.*, 2010; Charlson *et al.*, 2010, 2011; Morris *et al.*, 2013; Zemanick *et al.*, 2013; Tunney *et al.*, 2013; Zakharkina *et al.*, 2013; Goleva *et al.*, 2013; Segal *et al.*, 2013; Teo *et al.*, 2015; Bassis *et al.*, 2015; Cui *et al.*, 2015). Quelques travaux détaillent la flore fongique coprésente au niveau du tractus respiratoire (Delhaes *et al.*, 2012; Charlson *et al.*, 2012b; van Woerden *et al.*, 2013; Willger *et al.*, 2014); nous préciserons le mycobiote en seconde partie de notre introduction. La composition et la diversité de cet écosystème microbien sont uniques et caractéristiques pour chaque individu, variables selon son état de santé ou la présence de certaines maladies pulmonaires telle qu'un asthme, une broncho-pneumopathie chronique obstructive (BPCO) ou encore une mucoviscidose. Ce microbiote pulmonaire jouerait un rôle important dans la pathogénicité des maladies chroniques pulmonaires telles que la mucoviscidose. Les modifications du microbiote pulmonaire seraient corrélées à l'évolution clinique des patients. Par exemple, une perte de l'abondance et de la diversité (dysbiose) de cette communauté poly-microbienne est associée à une diminution de la fonction respiratoire du patient (Delhaes *et al.*, 2012; Madan *et al.*, 2012; Zemanick *et al.*, 2013; Harrison *et al.*, 2013). Ainsi, l'analyse NGS du microbiote pulmonaire et les nouvelles connaissances qu'elle génère suggèrent que nous devons revoir notre analyse de la pathogénèse et de la microbiologie des maladies pulmonaires chroniques en général et de la mucoviscidose en particulier. Comme le microbiome intestinal, le microbiome pulmonaire jouerait un rôle dans la régulation de la réponse immunologique. La dysbiose du microbiote pulmonaire pourrait causer un déséquilibre de l'homéostasie immunologique qui pourrait alors intervenir dans la genèse des

maladies respiratoires chroniques inflammatoires (pour revue (Marsland and Gollwitzer, 2014; Dickson *et al.*, 2015b).

D'autre part, alors que le microbiome bactérien et son rôle en pathogenèse sont largement étudiés, peu d'études ont porté sur la composante fongique (mycobiote/mycobiome) des communautés poly-microbiennes humaines. La faible abondance de champignons dans le microbiome humain les relègue sans doute à la partie dite « biosphère rare » (Huffnagle and Noverr, 2013). Cependant, cette biosphère rare semble avoir un impact important sur la santé de l'individu car elle peut servir de réservoir pour les agents pathogènes potentiels (tel que le genre *Candida*) ou les espèces clés qui ont un rôle essentiel dans le maintien des structure et fonction du microbiome humain (Huffnagle and Noverr, 2013). Enfin, le mycobiome peut être considéré comme un cofacteur d'inflammation en interaction avec les bactériome et virome (Marsland and Gollwitzer, 2014; Nguyen *et al.*, 2015).

A ce jour, quelques études se sont intéressées au mycobiote digestif ou cutané (pour revue (Underhill and Iliev, 2014; Marsland and Gollwitzer, 2014)) mais très peu ont abordé la composante fongique du microbiote pulmonaire (Charlson *et al.*, 2012b; Delhaes *et al.*, 2012; van Woerden *et al.*, 2013; Willger *et al.*, 2014). Pourtant plusieurs éléments démontrent l'importance de la flore fongique dans les pathologies respiratoires chroniques (notamment la mucoviscidose) :

-(i) Les spores (ou conidies) des champignons sont de petite taille (2 à 10µm), très facilement inhalées. Notre appareil respiratoire est donc exposé quotidiennement à des milliers de particules ou spores fongiques (un adulte respirant environ 15 m^3 d'air par jour contenant de 10^2 à 10^5 spores (Denning *et al.*, 2014)). Diverses composantes fongiques sont susceptibles d'entraîner des effets nocifs chez un individu exposé allant d'un effet toxique (tel que l'aflatoxine produite par *Aspergillus flavus*) à l'atopie.

-(ii) La relevance clinique d'une colonisation fongique, ou d'une sensibilisation (telle que l'aspergillose broncho-pulmonaire allergique ou ABPA) a été largement démontrée dans la mucoviscidose et l'asthme. Jusqu'à présent, l'étude de la colonisation fongique a porté sur les espèces des genres capables de pousser en condition standard de culture telles qu*'Aspergillus, Candida*, et à un degré moindre *Scedosporium* et *Exophiala*, ces différents agents fongiques étant susceptibles d'augmenter la mortalité et la morbidité des patients (Denning *et al.*, 2014; Touati *et al.*, 2014).

-(iii) Les traitements antibiotiques contre les infections bactériennes pourraient faciliter le développement des infections fongiques pulmonaires chez ces patients. De même, l'utilisation

de traitements immunosuppresseurs (corticostéroïdes au long court ou autres) favorise le développement d'infections fongiques invasives et graves.

-(iv) Enfin, les champignons sont capables d'interagir notamment avec des bactéries (pour revue voir Peleg *et al.*, 2010). Ils peuvent aussi former des biofilms et induire une résistance aux traitements anti-infectieux.

Actuellement, aucun individu ne semble pouvoir être « fungus free » (Huffnagle and Noverr, 2013). Aussi, le concept de mycobiote/mycobiome (incluant le mycobiote/mycobiome pulmonaire) représente un nouveau champ de recherche en pleine expansion que les techniques de séquençage à haut-débit rendent accessible (Huffnagle and Noverr, 2013; Underhill and Iliev, 2014; Marsland and Gollwitzer, 2014).

En 2012, notre équipe a commencé l'étude du mycobiote pulmonaire dans la mucoviscidose en utilisant la méthode de pyroséquençage (Delhaes *et al.*, 2012). Cette étude a confirmé l'existence d'un mycobiote riche et divers dans le poumon qui semble associé à l'évolution clinique des patients atteints de mucoviscidose.

Notre travail de thèse prend la suite de ce travail et s'inscrit dans les différents projets développés au sein de l'axe de recherche « Microbiote pro- et eucaryote pulmonaire » coordonné par le Pr Laurence Delhaes dans l'équipe Biologie et Diversité des Pathogènes Eucaryotes Emergeants (BDPEE) dirigée par le Dr Eric Viscogliosi. Il se focalise sur l'analyse NGS du microbiote pro- et eucaryotique respiratoire dans la mucoviscidose et notamment la comparaison de différentes approches méthodologiques en vue d'une optimisation et standardisation de la méthode. Notre travail est présenté en deux parties :

Dans un premier temps, nous présenterons une synthèse des connaissances actuelles d'une part des phénomènes de colonisations/infections fongiques chez les patients atteints de mucoviscidose et d'autre part dans le domaine du microbiote pulmonaire et surtout du mycobiote pulmonaire autour duquel notre équipe se focalise. Cette synthèse se traduit par une participation à la rédaction des 3 revues suivantes (cf. bilan en Annexe 1) :

- K Touati, **LDN Nguyen**, L Delhaes (2014). The airway colonization by opportunistic filamentous fungi in patients with cystic fibrosis: Recent Updates. *Curr Fungal Infect*, Vol 8, n° 4, p. 302–311. doi: 10.1007/s12281-014-0197-7.
- **LDN Nguyen**, E Viscogliosi and L Delhaes (2015). The lung mycobiome: an emerging field of the human respiratory microbiome. *Front. Microbiol*. 6:89. doi: 10.3389/fmicb.2015.00089.
- **LDN Nguyen**, L. Delhaes (2015). Un nouveau concept : Le mycobiome pulmonaire. *Médecine/Sciences*. Vol 31, n° 11, p. 945-947. doi: 10.1051/medsci/20153111002.

Dans un deuxième temps, nous avons cherché à mieux adapter l'approche de séquençage à haut-débit aux études du microbiote pro- et eucaryote pulmonaire sur des échantillons d'expectorations des malades atteints de mucoviscidose. En effet, le séquençage à haut-débit est une technique puissante mais pour laquelle des biais peuvent être introduits à de nombreuses étapes méthodologiques. Un des biais les plus importants est que l'approche NGS ne permet pas de différencier les microorganismes vivants des cellules mortes ou endommagées, ni de l'ADN extracellulaire. Dans le contexte du microbiote pulmonaire chez les patients atteints de mucoviscidose qui sont exposés souvent aux antibiotiques par voie intraveineuse à forte dose notamment en cas d'exacerbation pulmonaire aigüe et/ou d'infection chronique à *P. aeruginosa*, l'analyse par le séquençage à haut-débit pourrait évaluer incorrectement l'abondance et la diversité du microbiote pulmonaire. Le prétraitement des échantillons par propidium monoazide (PMA), qui permet de cibler sélectivement l'ADN des cellules vivantes (Nocker *et al.,* 2006), pourrait être une solution pour palier à cette limite (Rogers *et al.,* 2013). Notre étude avait donc comme objectif de déterminer si un prétraitement des échantillons avec PMA modifiait le microbiote pro- et eucaryote pulmonaire analysé par séquençage NGS. Cette étude est un projet collaboratif avec le service de Microbiologie de l'Université de Gand – Pr Mario Vanneechoutte et avec le consortium Pégase-GèneDiffusion à l'Institut Pasteur de Lille. Nous discutons l'intérêt et la relevance clinique de cette approche « PMA - NGS » permettant une quantification isolée des microorganismes vivants dans le contexte de la mucoviscidose.

In fine, l'objectif de notre travail de thèse a été de faire un état des lieux des connaissances actuelles des risques fongiques dans la mucoviscidose, des concepts de microbiote/mycobiote et leur application à l'analyse du poumon humain, puis de proposer une amélioration de l'approche méthodologique du microbiote/mycobiote pulmonaire par un prétraitement au PMA, ceci afin de mieux caractériser la flore poly-microbienne pulmonaire dans la mucoviscidose et sa relevance clinique.

IV. SYNTHESE BIBLIOGRAPHIQUE

A. Généralités sur la mucoviscidose

1. Epidémiologie

La mucoviscidose (ou fibrose kystique, "*cystic fibrosis*" en anglais) est la maladie génétique orpheline la plus fréquente dans la population caucasienne. Le sex ratio est 1 : 1 ; son incidence est variable en fonction des populations : entre 1/2500 à 1/4000 habitants pour les populations caucasiennes, estimée à 1/30000 pour les populations asiatiques et 1/15000 en Afrique (Ravilly *et al.*, 2007). Sa prévalence en Europe est estimée entre 8 et 12 cas pour 100 000 habitants. Selon les estimations de la « Cystic Fibrosis Foundation », le nombre des personnes atteintes de mucoviscidose dans le monde serait de 70 000 en 2012 dont 30 000 aux États-Unis (Cystic Fibrosis Foundation, 2012). En Europe (22 pays recensés), ce chiffre a été estimé à 32 248 en 2010 (Zolin *et al.*, 2010). En France, ce nombre s'éleverait à 6 196 en 2012, avec des prévalences variables selon les régions (Bellis *et al.*, 2013).

2. Pathogenèse et tableaux cliniques

La mucoviscidose est une maladie génétique autosomique récessive causée par mutation du gène « Cystic Fibrosis Transmembrane Regulator » (CFTR), localisé sur le bras long (q) du chromosome 7 (au locus 7q31.2). Ce gène code pour la protéine CFTR, qui est exprimée à la membrane apicale des épithéliums sécrétoires de plusieurs organes (sinus, glandes salivaires, thyroïde, poumon, canaux hépatiques, glandes pancréatiques, vésicule biliaire, intestin proximal, tubes collecteurs du rein, épididyme, canaux déférents, utérus, …). Cette protéine est un canal ionique dont le rôle est de réguler le transport des ions chlore à travers les épithéliums. Dans le cadre de la mucoviscidose, la mutation de ce gène entraine une altération de la protéine CFTR qui cause la dérégulation du transport d'ions de sodium et de chlorure (Figure 1). Il en résulte donc un déséquilibre des flux hydro-électrolytiques avec une concentration et une accumulation des sécrétions au niveau épithélial.

Dans ce tableau clinique, l'atteinte respiratoire est prédominante chez les patients. En effet, l'absence de CFTR entraine l'augmentation de la viscosité du mucus, ce qui diminue la capacité du système muco-ciliaire à éliminer les poussières et les microorganismes, et favorise l'apparition d'inflammations précoces devenant rapidement chroniques. La maladie s'accompagne souvent de toux, d'expectorations chroniques et d'obstructions bronchiques. Le cercle vicieux inflammation chronique et surinfection bactérienne (exacerbations infectieuses

répétées) aboutit à la fibrose sévère du tissu pulmonaire létale pour le patient. La maladie évolue donc progressivement vers l'insuffisance respiratoire.

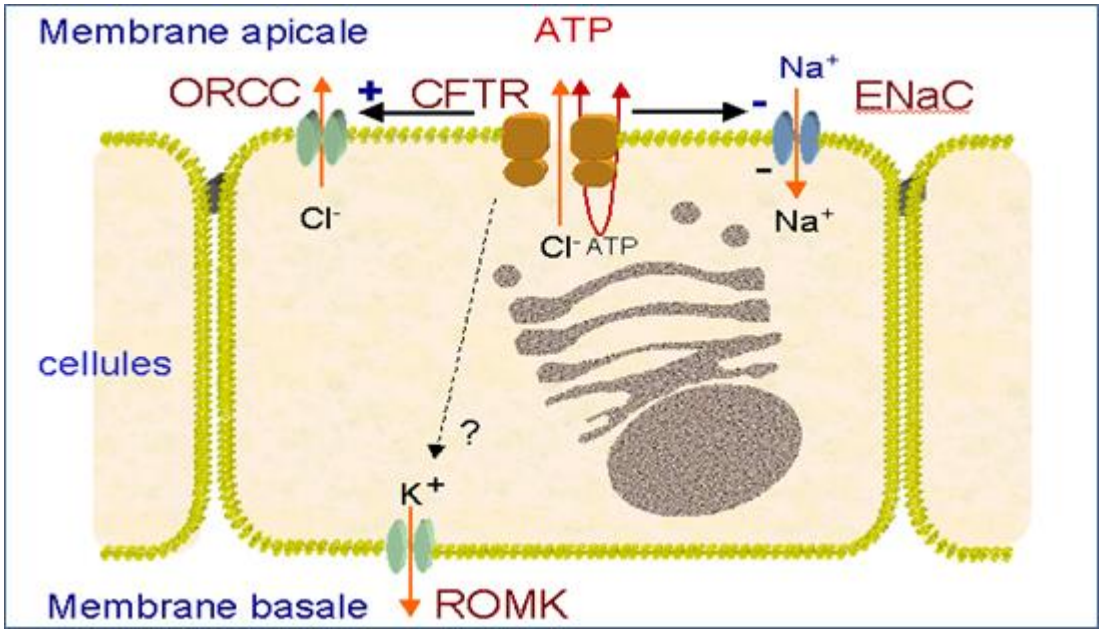

Figure 1 : Schéma des canaux régulés par la protéine CFTR (d'après Schwiebert et al., 1999)

3. La prise en charge du traitement

Actuellement, il n'existe pas de traitement curatif; le traitement proposé est symptomatique. L'objectif thérapeutique est une prise en charge multidisciplinaire dans les centres spécialisés (CRCM : centres de ressources et de compétences de la mucoviscidose), avec une coordination entre plusieurs spécialistes : pédiatre, pneumologue, kinésithérapeute, diététicien et psychologue, pour notamment ralentir la dégradation de la fonction respiratoire et améliorer la qualité de vie. La prise en charge de l'atteinte pulmonaire a deux objectifs principaux : améliorer la clairance muco-ciliaire et réduire les phénomènes de colonisation/infection bactérienne pulmonaire. Elle comprend la kinésithérapie respiratoire, l'antibiothérapie, des traitements par des bronchodilatateurs, des anti-inflammatoires et fluidifiants mucolytiques... La greffe pulmonaire est envisagée dans certains cas d'insuffisance respiratoire grave. Récemment, la prise en charge nutritionnelle associant un régime équilibré et hypercalorique et un apport en vitamines A, D, E et K, a été montrée comme primordiale dans le traitement global de cette maladie (Kerem *et al.*, 2005; Smyth *et al.*, 2014).

Aujourd'hui, grâce au dépistage génétique précoce (inclus en France dans le bilan néonatal systématique depuis 2002), à la surveillance multidisciplinaire des patients au sein du CRCM et surtout à l'amélioration de l'antibiothérapie, l'espérance de vie des patients a été améliorée

pour atteindre environ 46 ans aujourd'hui contre 7 ans dans les années soixante. Cependant la surveillance de l'état clinique notamment de la fonction respiratoire qui impacte directement le pronostic vital, reste très lourde et nécessite une prise en charge thérapeutique adaptée.

B. Problématiques des infections poly-microbiennes pulmonaires dans la mucoviscidose vers le concept du microbiote et mycobiote pulmonaire

Les infections chroniques pulmonaires représentent le problème majeur des malades atteints de la mucoviscidose. Plusieurs études montrent que le risque de mortalité est corrélé à la fonction respiratoire, évaluée essentiellement par le volume expiratoire maximal pendant la première seconde (VEMS1) (Milla and Warwick, 1998; Ratjen and McColley, 2012; Hulzebos *et al.*, 2014; Hayes *et al.*, 2015). La colonisation des voies respiratoires par les microorganismes (bactéries, champignons, virus) entraine notamment une réaction inflammatoire importante et des exacerbations aigües intercurrentes dont une des conséquences est la dégradation de la fonction respiratoire pouvant mettre en jeu le pronostic vital du patient (Sanders *et al.*, 2011; Ratjen and McColley, 2012). La physiopathologie de l'infection pulmonaire est ici complexe, due à un environnement pulmonaire unique où clearance mucociliaire altérée et adhésion microbienne au mucus épais augmentée se conjuguent et favorisent la persistance de germes sur de nombreuses années, ce qui aboutit à une inflammation chronique rythmée par des surinfections broncho-pulmonaires d'origine bactérienne et/ou fongique. Ces infections microbiennes restent à ce jour difficile à traiter, en raison d'une diffusion antibiotique limitée à cause de l'épaisse couche du mucus et/ou l'apparition de souches bactériennes multi-résistantes dans les poumons des malades et/ou le développement de biofilm (Aaron *et al.*, 2005; Sherrard *et al.*, 2014; Waters and Ratjen, 2015).

1. Infections bactériennes

Les principales bactéries qui infectent les voies aériennes de façon chronique détectées par les méthodes conventionnelles sont *S. aureus*, *H. influenzae* et *P. aeruginosa*. Plus récemment, d'autres germes ont émergé notamment *Stenotrophomonas maltophilia*, *Achromobacter xylosidans* et le complexe d'espèces *Burkholderia cepacia* (Ciofu *et al.*, 2013).

Chez les patients pédiatriques, *S. aureus* est la bactérie la plus fréquemment isolée par la méthode de culture (Razvi *et al.*, 2009). Avec l'amélioration de l'antibiothérapie, la mortalité

causée par *S. aureus* a diminué, mais *S. aureus* reste toujours un pathogène prédominant chez les patients jeunes (depuis la naissance à l'adolescence).

Chez les patients adultes, les infections pulmonaires chroniques à *P. aeruginosa* représentent plus de 80% des infections bactériennes et sont une cause majeure de mortalité (Ratjen and McColley, 2012; Ciofu *et al.*, 2013). Sous l'effet du stress local pulmonaire (réponse immune de l'hôte et antibiothérapie), *P. aeruginosa* a développé des stratégies de survie et de prolifération dans le poumon des patients atteints de mucoviscidose (Sousa and Pereira, 2014). Il est ainsi capable d'évoluer à partir d'un organisme non mucoïde mobile et virulent vers un organisme mucoïde immobile et relativement non virulent qui pourrait être mieux adapté à la survie dans les voies aériennes de ces patients. Cette transition (organisme non mucoïde *versus* organisme mucoïde) est due essentiellement à des mutations inactivant le gène *mucA*, qui régule la production d'alginate (Ciofu *et al.*, 2010). Si ces bactéries apparaissent sur gélose comme des colonies muqueuses, dans le poumon ces souches pourraient se développer sous forme de biofilms avec une sécrétion de molécules du «quorum-sensing» au niveau de la couche de mucus et une capacité à résister à la phagocytose, à la réponse immune de l'hôte et au traitement antibiotique (Bjarnsholt *et al.*, 2009; Høiby *et al.*, 2010). Bien que ces souches mucoïdes soient relativement non virulentes, elles induisent une réponse inflammatoire à bas bruit chronique. L'apparition de *P. aeruginosa* bouleverse donc la prise en charge des patients sur le plan microbien : les traitements d'antibiotiques intensifs et précoces contre l'infection à *P. aeruginosa* et son passage à la chronicité ont montré une augmentation de l'espérance de vie des patients (Høiby *et al.*, 2005; Hansen *et al.*, 2008; Geller, 2009).

2. Infections fongiques

Pendant plusieurs années, l'accent a été mis sur des infections bactériennes chroniques des voies respiratoires des patients. Jusqu'à récemment, le manque de technique de culture standardisée entre les laboratoires (prétraitement des échantillons, choix des milieux de culture, température et durée des incubations, méthodes d'identification des micromycètes…) pour isoler les champignons dans les échantillons respiratoires limitaient probablement nos connaissances des pathogènes fongiques et de leur rôle dans la dégradation de la fonction respiratoire des patients. Cependant, de nombreuses espèces fongiques émergentes ou ré-émergentes sont de plus en plus isolées (Pihet *et al.*, 2009). En particulier, récemment les champignons filamenteux tels que *A. fumigatus*, *A. flavus*, *Aspergillus terreus*, les espèces du complexe *Pseudallescheria boydii*/*Scedosporium apiospermum, Scedosporium prolificans*

[récemment renommé *Lomentospora prolificans]* et *Exophiala dermatitidis* sont fréquemment isolés et mis en cause dans l'altération de la fonction respiratoire des patients. Ils peuvent également être responsables d'infections invasives. Plus récemment, d'autres champignons ont été identifiés tels que *Rasamsonia argillacea*, *Acrophialophora fusispora* et décrits comme des agents émergents également potentiellement responsables des infections chroniques chez les patients atteints de mucoviscidose. De plus, les colonisations/infections fongiques ont été associées à la dégradation de la fonction respiratoire chez les patients atteints de mucoviscidose (Horré *et al.*, 2010; Delhaes *et al.*, 2012; Middleton *et al.*, 2013; Touati *et al.*, 2014; Nguyen *et al.*, 2015).

La revue suivante (Article 1) à laquelle j'ai participé avec le Dr Kada Touati et le Pr Laurence Delhaes, présente l'ensemble des connaissances récentes sur l'épidémiologie, l'écologie et la physiopathologie des champignons filamenteux capables de coloniser et/ou d'infecter les voies respiratoires des patients et qui pourraient être responsables du déclin de la fonction pulmonaire. Nous y résumons les caractéristiques principales non seulement des espèces fongiques très répandues et connues comme *Aspergillus* et *Scedosporium* (Figures 2 et 3), mais aussi, des champignons décrits plus récemment, tels que *R. argillacea*. En même temps, nous discutons les écueils actuels dans le suivi et l'interprétation des phénomènes de colonisation/infection fongique dans la population de patients atteints de la mucoviscidose. En particulier dans le contexte des infections poly-microbiennes chez les malades atteints de la mucoviscidose, nous mettons l'accent sur la nécessité de mieux caractériser le rôle des champignons dans la pathogénèse de la maladie pulmonaire mucoviscidosique et leur prise en charge dans ce contexte. Dans cette revue, j'ai contribué notamment au développement du concept du microbiote pro- et eucaryote pulmonaire et à la bibliographie.

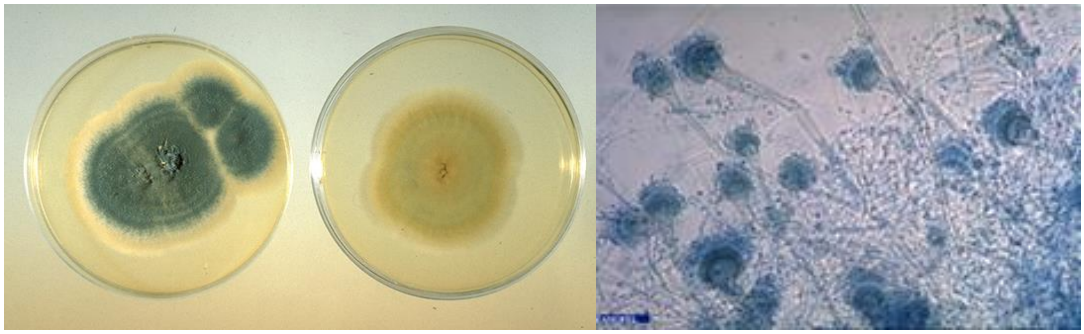

Figure 2 : Aspect d'*Aspergillus fumigatus* sur milieu Sabouraud et au microscope (source : ANOFEL4)

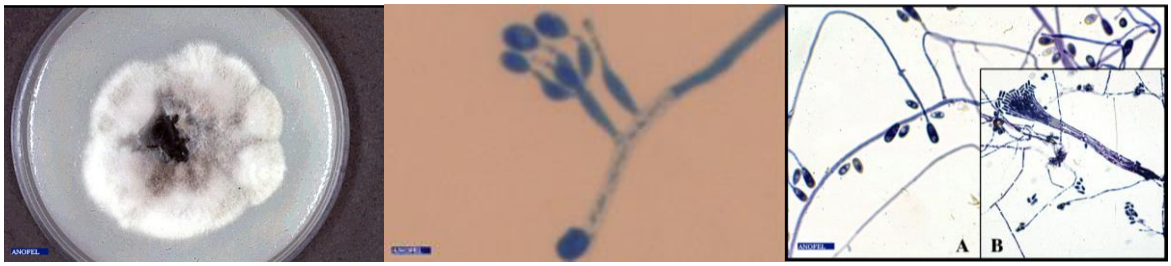

Figure 3 : Aspect de *Scedosporium prolificans* et de *Scedosporium apiospermum* sur milieu Sabouraud et au microscope (source: ANOFEL4)

<u>Article 1 :</u> The airway colonization by opportunistic filamentous fungi in patients with cystic fibrosis: Recent updates

Publication dans le journal *Current Fungal Infection Reports*, Vol 8, n° 4, p. 302–311. doi: 10.1007/s12281-014-0197-7.

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<u>Article 1:</u> The airway colonization by opportunistic filamentous fungi in patients with cystic fibrosis: Recent updates

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Introduction

Cystic fibrosis (CF) is the most common genetic inherited disease in the European white population, and based on the number of patients, is the third most common orphan disease. CF is caused by mutations in the CFTR gene that encodes a chloride channel involved in electrolytic exchanges through the plasma membrane of epithelial cells. In the respiratory tract, mutations in CFTR result in a defective mucociliary clearance and a thickening of the bronchial mucus. These abnormal airway conditions facilitate the entrapment of the inhaled bacterial and fungal conidia, and provide a suitable environment for growth of microorganisms. This, in turn, leads to respiratory infections and inflammatory reactions which, together with the microorganisms, contribute to the progressive deterioration of lung function. Using conventional microbiological culture methods, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Burkholderia cepacia* complex have been identified as the principle pathogenic bacteria in adult CF pulmonary infection that cause recurrent exacerbations of the pulmonary disease and often determine the vital prognosis of patients [1, 2]. As such, over recent decades considerable attention has been paid to their prevention and treatment by antibiotics, which has improved patient management and resulted in a significant increase in the life expectancy of patients [3, 4]. As a consequence, the respiratory tracts of CF patients are at increasingly high risk of colonization and/or infection by several fungi [4, 5]. In this context, more and more attention has been drawn toward filamentous fungi, as well as certain yeasts reaching the status of emerging or re-emerging microorganisms [6, 7]. While recent studies have shown an association between *Aspergillus fumigatus* colonization and poorer lung function, as well as negative prognostic value of an allergic bronchopulmonary aspergillosis (ABPA) status in CF [8, 9, 10•], fungal isolation in respiratory secretions remains poorly studied in the context of CF, and continues to present a dilemma for the clinician in terms of therapeutic management [11]. In this review, we provide a summary and

discussion of recent insights into the epidemiology, ecology, and physiopathology of the filamentous fungi that are able to colonize and/ or infect the airways of patients with CF, and that which may be responsible for accelerated lung function decline [8, 9, 10•, 12, 13]. We focus not only on highly prevalent species such as *Aspergillus*, *Scedosporium,* and *Exophiala*, but also on more recently described fungi such as *Rasamsonia argillacea* [7, 12–15]. With this insight into the fungal community, we propose to ascertain the worldwide occurrence of fungal colonization in the respiratory tract (including estimated ABPA rate) in CF. Our focus is on the role of the fungus in the context of improving survival (especially with regard to lung transplantation), taking into account the new concept of lung mycobiota. Further studies are warranted to evaluate the role of fungi in CF lung disease and to determine guidelines for therapeutic management.

CF: An old disease, with survival improved by new therapies targeted at fungal colonization/infection

CF is the most common fatal genetic disease (autosomal recessive transmission without sex ratio) in white populations. It has likely been noted in various definitions in the literature since the days of antiquity, described in Northern Europe in the 19th century as "misery to the child who leaves a salty taste when he kisses: he will die soon." In 2012, the estimated number of worldwide cases of CF reached 70,000, including 30,000 in the United States, according to recent Cystic Fibrosis Foundation (CFF) estimates [16]. In Europe, the number of individuals with CF was estimated at over 32,000 in 2010 [17], and in Australia at 3,156 people [18] (Fig. 1). The overall incidence of CF in white populations is thought to be between 1/2,500 and 1/4,000; it is lower for Asian and African populations (Fig. 1) [19–24, 25••, 29]. In recent years, the life expectancy of patients with CF has increased significantly [3, 16, 17, 19], for example, from 31 years in 2002 to 41 years in 2012 in the U.S. [16]. This improvement is seen as a result of the creation of CF treatment centers staffed with multidisciplinary teams, offering of a more effective treatment paradigm for the disease [3, 4]. Nevertheless, CF remains virtually incurable, a disease in which abnormal mucociliary function and local immunogenic injury promote fungal development in the lungs and promote bacterial colonization and infection. Patients are at risk of death from respiratory failure related to acute pulmonary exacerbations, and for which prompt aggressive treatment with antibiotics is recommended [3]. Antibiotic therapy for airway infection in CF is now well-documented, with consensus guidelines in North America and Europe [3]. Although long-term antibiotic therapy using inhaled and/or oral broad-spectrum antibiotics has been associated with fungal colonization in patients with CF [26, 27, 30], a recent study has shown

a significant reduction in the presence of *A. fumigatus* after a short-term intravenous antibiotic regimen against *P. aeruginosa* [31]. Approximately 10 % to 20 % of patients who present with pulmonary exacerbation do not respond or respond only partially to intravenous antibiotic treatment, which usually targets the dominant bacteria identified by cultures [2, 3, 28, 32]. In these populations, the risk of failure to recover lung function has been associated with CF-related diabetes, pancreatic insufficiency, malnourishment, liver disease, *P. aeruginosa* multi- or pan-drug-resistant isolates, chronic *P. aeruginosa* infection, methicillin-resistant *S. aureus*, *B. cepacia* complex infection, and/or ABPA [28, 32, 33]. An increase in the prevalence of fungal infections has been noted with respect to rising numbers of hospital admissions and/or with a decreased lung function [10•, 12, 30]. As such, assessment for the presence of fungi in respiratory secretions, in addition to ABPA criteria, is now considered as essential in the follow-up of patients with CF [3]. New therapies that target the global CFTR protein deficiency – i.e., organ transplantation, and more recently CFTR potentiators such as ivacaftor (Kalydeco®, Vertex Pharmaceuticals) – are expected to have an impact on rates of morbidity and mortality with regard to fungal-related pulmonary disorders in patients with CF. Treatment with CFTR potentiators requires further study to provide data on its long-term effectiveness [34]. Transplantation, and particularly lung transplantation, is increasingly common (Fig. 1), and is associated with the risk of developing invasive mycosis [16–18, 35–37]. The incidence of invasive aspergillosis (IA) among lung transplant patients has been reported between 10 % and 22.5 % [35, 37, 38], and is also associated with higher mortality rates in CF [35, 37]. Overall, there is a growing body of evidence that the fungi, and filamentous fungi in particular (including *Exophiala* species, which are dimorphic fungi, able to grow as black yeast at body temperature and as filamentous fungi at room temperature) present in the lungs of patients with CF are pathogens rather than spectator microorganisms. As they able to confer a large spectrum of disease (sensitization, ABPA, and/or invasive mycosis), in the following two sections we will review the fungal species recently reported in CF and discuss their potential pathogenicity.

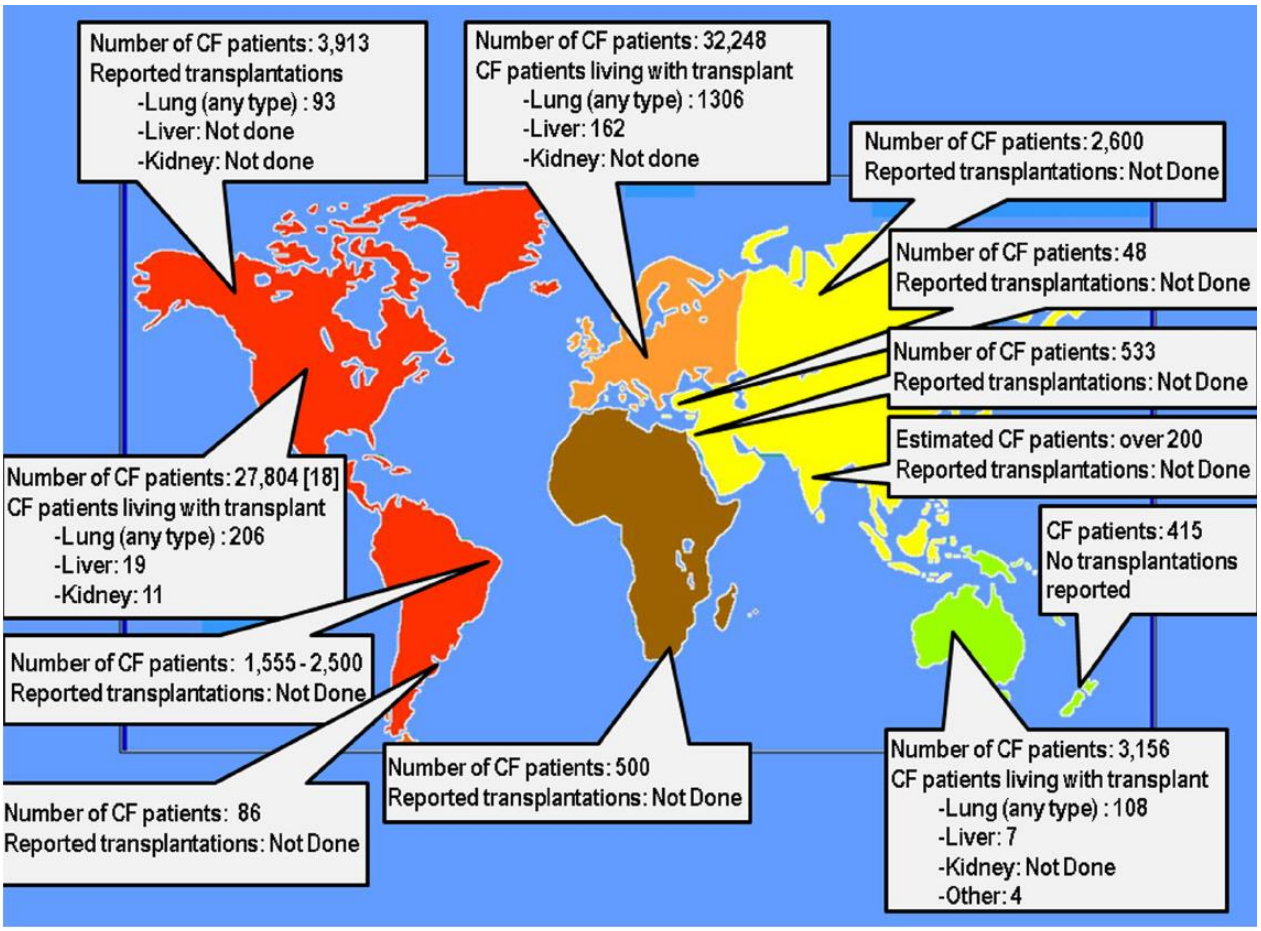

Fig. 1: Worldwide distribution of patients diagnosed with CF and estimated number of CF patients living with transplant (data collected from [18–24, 25••, 26–28]

Aspergillus fumigatus: The tree that hides the forest

Aspergillus species are ubiquitous molds, present everywhere in soil, air, vegetation, food, and indoor and outdoor human environments. While over 200 species have been described, only about 20 are pathogenic to humans [39]. The *Aspergillus* species, particularly *A. fumigatus*, are those most frequently isolated in the respiratory tracts of patients with inflammatory chronic pulmonary disease such as asthma or CF [reviewed in 40, 41]. The prevalence of *A. fumigatus* ranges from 5 % to almost 90 % [10•, 15, 23, 27, 30, 32, 42–55] (Table 1). *Aspergillus* species are associated with significant morbidity, and are associated with diseases that likely remain underdiagnosed [25••]. They manifest clinically in various ways, ranging from infection to ABPA (reviewed in [39]). While ABPA is the most common clinical manifestation in CF, IA and aspergilloma have also been reported [39, 40]. Aspergilloma, which is observed rarely in CF, requires a pre-existing lung cavity to develop, is difficult to treat, and may require surgical resection or long-term antifungal treatment [39]. Two entities of invasive disease have been differentiated and proposed in CF: invasive

aspergillosis (IA), occurring in the setting of immunosuppressive states during transplantation, and invasive pulmonary aspergillosis (IPA) in non-transplant CF patients with lung impairments. IPA is the most frequently observed pathology in CF, although invasive diseases as a whole remain highly rare in non-transplant CF patients. IA has been recently associated with a fourfold higher risk of occurrence in the case of colonization with *A. fumigatus* [38], and its management remains a diagnostic and therapeutic challenge. Recent studies of *Aspergillus* sensitization, ABPA, and *Aspergillus* bronchitis (an entity described in CF patients with decreased FEV1 [25••, 56]) have indicated that they are underdiagnosed in the worldwide CF adult population [25••]. The estimated numbers of adult CF patients with Aspergillus sensitization, ABPA, and Aspergillus bronchitis have been estimated at 5,506, 6,675, and 11,314, respectively [25••]. ABPA represents a severe complication in patients suffering from CF. It is difficult to diagnose due to its overlapping clinical and radiological features with those of CF exacerbations. Since early treatment of ABPA has been found to reduce symptoms, improve lung function, and prevent long-term damage such as bronchiectasis and fibrosis, the prompt diagnosis of ABPA is imperative. New criteria based on established serological tests (IgG and IgE serum levels) combined with real-time PCR and levels of galactomannan in sputum have recently been proposed in order to classify patients with CF into three groups of aspergillosis, which may be helpful for the management and follow-up of *Aspergillus* disease in CF [56, 57]. In addition to sporadic use of voriconazole, the recommended protocols to treat ABPA in CF are based on itraconazole to target *A. fumigatus*, which then raises the more general question of developing azole resistance [48, 58–60]. An ongoing prospective randomized study, ATCF (Azole Therapy in Cystic Fibrosis [EudraCT: 2011-005799-41]) coordinated by Gangneux and Denning is addressing (i) the efficacy of itraconazole and voriconazole in CF patients, (ii) the relationship between plasma azole concentrations and the clinical response, and (iii) the relationship between fungal molecular typing and in vitro chemosensitivity to different antifungal agents. This study may pave the way for new therapeutic guidelines. Other species – *Aspergillus flavus*, *Aspergillus terreus*, *Aspergillus niger*, or *Aspergillus nidulans* – are capable of colonizing the respiratory tract in CF patients, in frequencies ranging from 11 % to less than 0.1 % (Table 1). They have been reported in ABPA cases as well as invasive aspergillosis [15, 39, 58, 61]. Unusual *Aspergillus* species such as *Aspergillus lentulus* or *Neosartorya pseudofischeri*, which are known to exhibit low susceptibility to amphotericin B and/or voriconazole, have also been described in CF [58, 62]. The frequency of isolation of *Aspergillus* species in CF, and particularly *A. fumigatus*, has varied among recent published data (Table 1), increasing

significantly with patient age, use of antibiotics, and decreased lung function [10•, 27, 30]. In addition, *Aspergillus*-persistent carriage and sensitization have been shown to exhibit an independent effect on lung function in CF [10•], and *Aspergillus* sensitization has also been associated with decreased lung function [63]. Genotypic studies of *Aspergillus* species (largely *A. fumigatus*) have been conducted in order to clarify the epidemiology of aspergillosis and to identify potential sources, transmission routes, and colonization patterns of *Aspergillus* isolates [64]. As was recently shown with the *A. terreus* isolate [61], different profiles of genotypes can be observed among the species: (i) transient colonization by a single genotype, (ii) chronic colonization by several genotypes, and (iii) chronic colonization by one or a few dominant genotype(s). In fact, the respiratory tract of CF patients is more frequently colonized by multiple *Aspergillus* strains, whereas only a single genotype is identified from deep organs of patients with invasive disease. Molecular typing methods and results have been exhaustively reviewed elsewhere [64]. Recent findings have underscored the complex interplay between the respiratory epithelium and the host response in the presence of inhaled spores. A recent review looked at the classical major components of the innate immune system as well as the cytokine host response involved in recognition and removal of A. fumigatus [39]. In addition to its thermotolerance and its propensity to disseminate in immunocompromised hosts (due to the small size of conidia, 2–3.5 μm, that may be inhaled into the alveoli), *A. fumigatus* is able to modulate the inflammatory response in epithelial cells [63, 65], due to the compounds of its fungal surface cell (reviewed in [66]). It has also been associated with significant local inflammatory response in young CF patients [67], a fact that is highly supportive of the use of specific surveillance for such filamentous fungus.

Table 1 Worldwide frequency of isolation of *Aspergillus* spp., *Scedosporium* sp., and *Exophiala dermatitidis* in CF respiratory tract obtained from bibliography from 2010 to date.

Continents and Countries (Number of CF patients) [Bibliography]	**Frequency of fungal Isolation (%)**								
	***Aspergillus* species**						**Non-*Aspergillus* species**		
	A. fumigatus	***A. flavus***	***A. terreus***	***A. niger***	**Other species**		**Species of the complex *P. boydii / S. apiospermum***	***S. prolificans***	***E. dermatitidis***
Europe									
Austria (113) [15]	34	3	2	ND*			2	ND	3
Belgium (154) [45]	7.8	0.6					ND	ND	5.9
Denmark (148) [46]	28.8	2.7	0.7	0.7	0.0		*S. apiospermum* 0.7 *S. aurantiacum* 0.7	0.7	ND
Denmark (287) [47]	13.6	4.1-4.4**	1.9-2.6	4.7-0.8	0-1.4		< 1	ND	ND
France (251) [10]	27.1	ND					ND	ND	ND
France (249) [48]	52.6	ND					ND	ND	ND
France (201) [49]	56.7	10.4					3.4	ND	ND
France (291)*** [42]	45.4	3.4	0.0	1.7	2.1		3.8	0.0	0.0
Germany (81) [44]	*Aspergillus* sp.: 47.8						4.7	0.7	1.6
Ireland (77) [43]	5.2	3.9					3.9	ND	3.9
Italia (1837) [50]	11.8	3.0	2.4	0.6	ND		1.9	ND	0.05

The Netherlands (259) [28]	23.5	ND					ND	ND	ND
Sweden (97) [51]	10.4	ND					ND	ND	19
UK (51) [32]	37.0	ND					ND	ND	ND
UK (69) [42]	43.5	2.9	5.8	1.4	1.4		1.4	0.0	0.0
UK (36) [42]	88.9	5.6	11.1	0.0	0.0		8.3	0.0	1.4
Middle East									
Turkey (48) [23]	10.4	8.3					ND	ND	ND
Israel (468) [52][£]	*Aspergillus* sp.: 35.5						ND	ND	ND
North America									
USA (614) [29]	36.3	26.1					2.3[£££]		5.0[£££]
South America									
Brazil (74) [53][£]	*Aspergillus* sp.: 5.6						ND	ND	ND
Asia									
India (41) [54]	18.2	ND					ND	ND	ND
Oceania									
Australia (72) [55]	66.7	10.1	ND	ND	8.7		*S. aurantiacum* 5.8 Other complex species: 5.8	5.8	0.0

*ND: Not done; **Data analysis was performed during two separate periods (2007 and 2009); ***French data from Angers, Lille and Rouen reported in 2006 (Table 2 in [41]) have been included together; [£] Only data published before 2010 were available; [£££] Reported as *Scedosporium* spp; [£££] Reported as other filamentous species including *Alternaria, Curvularia, Paecilomyces, Penicillium,* and *Exophiala* species.

Fungal risk in CF and recent findings on non-*Aspergillus* species

Candida is the most commonly isolated yeast in the respiratory tracts of patients with CF. It is considered as a commensal microorganism belonging to the oral microbial community regardless of the fact that *Candida albicans* has been associated with a decline in FEV1 [12, 15, 23, 30, 42–44, 68]. In addition to *A. fumigatus*, other moulds such as *Scedosporium* sp. have been reported, at times with higher frequency than those of non-fumigatus *Aspergillus* (Table 1) [42, 43, 45, 50, 69]. These wide ranges are likely as a result of one or more of the following points: (i) the non-standardized protocol used to isolate fungi from samples (protocols are different according to addition or absence of pretreatment step, number and choice of media, incubation temperature, culture duration, and methods used to identify isolated fungi), (ii) the types of sampling used (nasal swab, sputum, bronchoalveolar lavage), (iii) the age of CF patients studied (adults or children), and (iv) the characteristics of local climatic conditions [5, 11, 15, 25••, 42, 70–72]. Although mycological culture methods vary from one center to another and suffer from poor sensitivity [38, 57, 71], isolation of fungi such as species of the *Pseudallescheria boydii*/*Scedosporium apiospermum* complex or *Exophiala* sp. is remarkably more common, and seems to be associated with older age [27, 30, 42], decreased lung function [10•, 30, 69], and/or use of antibiotics [27, 30, 53]. In this context, filamentous fungi are by far the most pathogenic micromycetes frequently isolated, but black yeasts (*Exophiala* sp.) are also reported. *Scedosporium* species are ranked as the second most common genus associated with CF after *Aspergillus* (Table 1). It formerly comprised two species, *S. prolificans* and *S. apiospermum*, but the latter has recently emerged as the *P. boydii*/*S. apiospermum* complex initially described by Gilgado et al. [73–75]. The species from the *P. boydii*/ *S. apiospermum* complex are the more prevalent in CF (Table 1)[74], but *S. prolificans* is the more lethal due to its ability to disseminate and its antifungal resistance [6]. Chronic colonization and fatal outcome with *S. apiospermum* have also been reported [74, 76]. In a retrospective single-center study, it appeared to be responsible for the development of ABPA-like symptoms, or ABPM (allergic bronchopulmonary mycosis) [49]. Among the newly described species of the *P. boydii*/*S. apiospermum* complex, *Scedosporium aurantiacum* has been more frequently isolated from sputum samples of CF patients in France and in Australia [55, 56, 77] (Table 1). These differences in rates of recovery are likely due to methodology discrepancies, since species of the *P. boydii*/*S. apiospermum* complex are known to be optimally detected with selective medium and a long period (up to 7–11 days) of medium incubation [44, 55, 71, 77, 78]. The risk factors for *Scedosporium* acquisition in CF remain poorly identified. Co-colonization of *Pseudallescheria*/*Scedosporium* species with

other moulds, primarily *A. fumigatus*, has been described [49, 55, 71]. Univariate analysis has shown that bacterial colonization and antimicrobial exposure likely influence *Scedosporium* colonization [55]. In Australia and France, soil sampling revealed an abundance of *Pseudallescheria*/*Scedosporium* species, mostly in areas associated with human activity [77–79], which is consistent with the high prevalence of *Scedosporium* infection and colonization previously described in CF patients [80]. In Australia, *S. aurantiacum* was the most frequently isolated species, at 54.6 %, followed by *S. prolificans* (43 %), *P. boydii* (2.1 %), and *S. dehoogii* (0.3 %) [80]. Recent molecular studies have suggested that most patients were chronically infected with a single strain, with no sharing of similar strains [77, 81]. Among the various *Exophiala* species isolated from respiratory tracts of CF patients, *Exophiala dermatitidis* is most frequently observed [82], and appears to be more frequent in the north of Europe (Table 1). In a Swedish study [51], this black yeast was recovered more frequently than *A. fumigatus* from the sputa of patients with CF (Table 1). With regard to associated risk factors, *E. dermatitidis* culture-positive patients tended to be significantly older than 12 years, pancreatic insufficient, homozygous for the F508del mutation, and colonized with *A. fumigatus* [45, 51, 69]. In a recent study, antibody production (IgG) against *E. dermatitidis* was significantly associated with an inflammatory response (higher white blood cell count) and decreased respiratory function (lower value of FEV1% predicted) [69]. In terms of molecular analysis, most of the *Exophiala* isolates were grouped according to the patient origin, with no association with respect to the geographical origin of the isolates, the isolation date, or antifungal susceptibility [82]. *Rasamsonia argillacea*, previously known as *Geosmithia argillacea*, is a filamentous fungus similar to the *Penicillium* species and genus, which can lead to misidentification, and certainly may contribute to its underestimation [14]. Recent molecular analyses have led to the identification of a species complex that requires further studies [7, 13–15, 83]. Chronic colonization with *R. argillacea* has recently been reported in France, Italy, and the UK [7, 13–15, 50, 83]. While the number of cases is low, predisposing factors have been identified and include homozygosis for the F508del mutation and previous use of azole to manage mould colonization [83]. *R. argillacea* showed a significant sensitivity to echinocandin during in vitro susceptibility tests [13, 14]. Notwithstanding the increasingly common isolation of *R. argillacea* in the respiratory tract, its pathogenicity remains poorly demonstrated [14]. Isolation of *Acrophialophora fusispora* in the context of CF has not been reported since 2005. Since this mould is as difficult to identify as *R. argillacea*, even in expert mycology departments, its incidence is likely underreported, and hence its clinical relevance poorly documented. The fungus has been reported to be

responsible for keratitis and pulmonary infection in non-immunocompromised adults, and was noted to be associated with a brain abscess in a child with acute lymphoblastic leukemia. In patients with CF, chronic colonization with *A. fusispora* may contribute to progressive pulmonary impairment by promoting local inflammation [84].

Microbial diversity in CF airways: From in vitro interactions among microorganisms to clinical insights in CF lung microbiota

A number of studies have shown that the CF airways are colonized by diverse polymicrobial communities, recently referred to as "lung microbiota," with bacteria, fungi, and viruses all present and potentially contributing to infection and inflammation [85–98]. While microbiological cultures are useful for the diagnosis of bacterial and/or fungal infections, they are less suitable for identifying co-infections and dynamics of microbial populations, as viral microorganisms are usually not diagnosed. Yet such organisms have become increasingly recognized as important agents, especially in CF pulmonary exacerbations [91–94]. Therefore, determining the microbial composition of the upper airways that characterize each patient becomes important, as well as determining its microbial evolution during CF pulmonary disease. In addition, significant associations (co-colonization or exclusion) between fungi or between fungus and bacteria have been described in the context of CF [44, 51, 55, 99], and highlight the aptitude of the fungus to interact with other members of the microbial community colonizing the airways of patients with CF, and consequently contributing to the alteration of lung function. Bronchoalveolar lavages of children with CF that grew more than one microorganism have also been associated with greater inflammatory levels [67]. Such interactions, especially between *A. fumigatus* and *P. aeruginosa*, have also been demonstrated in vitro. These two organisms are able to produce biofilm, and involve quorum-sensing molecules. Although the development of numerous molecular techniques has provided for accurate fungal detection and classification, few of these techniques have addressed the polymicrobial composition of the fungal community in CF [85–90, 92–98]. Advanced techniques such as deep-sequencing methods, which are able to massively identify microbial sequences (thousands of sequences in a few hours), have provided new insights into the depth and breadth of lung microbiota, particularly in CF. The vast majority of the published data have explored the diversity of bacterial communities [93–97], with only a few studies focusing on viruses and phages [92] or fungi [86, 87, 89, 96]. On the whole, published results are promising: they uncover the presence of a bacterial com- munity present in both healthy and pathologic lungs [88, 95], which may represent a subpopulation of the microbiota. The whole flora is able to evolve according to the primary pulmonary disease (COPD,

asthma, bronchiectasis, or CF) and based on the presence of absence of acute exacerbation [92–94]. In fact, each microbiota has its own composition and evolution that is unique and specific to the patient, and which may play a role in the deterioration of lung function. High-throughput technologies provide the opportunity to simultaneously analyze the whole (bacteria, viruses, and fungi) microbial community without a priori knowledge of existing microorganisms, and consequently represent the most promising investigational strategy in the context of pulmonary chronic diseases such as CF.

With respect to fungal diversity or mycobiota, these new approaches have recently allowed us to identify a higher number of fungal species versus conventional culture-based methods, including species previously undescribed in the context of CF such as *Malassezia* species [87, 89, 90]. These are lipophilic yeasts, difficult to grow in standard culture media, and commensally found in the human skin. Their reduced abundance in respiratory samples was recently correlated to a decline in CF pulmonary function [88]. This result is consistent with the complex bacterial and fungal diversity recently reported using the pyrosequencing method, in which more than 60 % of the species or genera had not been identified in cultures [87]. Strikingly, the diversity and species richness of fungal and bacterial communities observed was significantly lower in patients with decreased lung function and poor clinical status/outcome [86, 87]. Whether all members of this fungal community play a direct or indirect role in pulmonary decline has yet to be fully elucidated, especially regarding the cooperative, competitive, and adaptive interactions of microorganisms isolated in the CF lung microbiome, as recently proposed [98]. Further, larger studies based on deep-sequencing approaches are now warranted to address mycobiota and microbiome in the context of CF. These studies should take into account newly proposed criteria that could improve clinical classification of aspergillosis in CF [31, 57].

Conclusions: Implications in the microbiological diagnosis of fungi as non-bystanders in the CF lung

With the exception of *A. fumigatus*, for which the role is well-documented, the pathogenic role of fungi colonizing the CF respiratory tract is still a matter of debate. However, all represent true opportunist pathogens. They share biological features that are essential to colonize and infect lungs: thermotolerance, a capacity to produce biofilm, and an ability to disseminate and/or to resist to various antifungals, especially the azole drugs [14, 48, 59, 60, 71]. As such, greater insight into their clinical relevance (such as an increasing prevalence associated with older age of CF patients and/or a decreased lung function) has emerged within the past decade [10•, 27, 30, 42, 63, 69]. From a practical point of view, greater attention

should be given to any chronic colonization by filamentous fungi, especially through the use of specific procedures for mycological analysis of CF respiratory samples. A recent international initiative, the MucoFong International Project (MFIP) [100], designed to compare the performance of different media used for fungal cultures and develop a standardized approach for the mycological ex- amination of sputum samples from CF patients, will facilitate the establishment of unique, or at least comparable, mycological procedures. Briefly, MFIP study was organized within the framework of our ECMM/ISHAM Working Group on Filamentous Fungi and Chronic Respiratory Infections in Cystic Fibrosis. It is based on a unique protocol shared by all participating centers [100]. This protocol has been collectively validated according to the MFIP questionnaire that we organized as a preliminary step to design the MFIP study (i.e., each center was allowed during the same three-month period of time to include 25–30 sputa from CF patients, used the same procedure and the same mycological media to isolate fungi, and summarized its results onto a prepared Excel table; all media were prepared in Lille and dispatched to the participating centers; results were synthesized and analyzed in Lille). MFIP protocol includes pretreatment (to homogenize) of sputum samples that improved the isolation of non-*Aspergillus* species [15]. Given the emergence of resistant species (primarily azole-resistant *Aspergillus* isolates), their contribution to ABPM disease, and the importance of these fungi as potential sources of infection after lung transplantation, azole-susceptibility testing should also be performed on all fungal isolates chronically colonizing the airways of patients with CF (at least all *Aspergillus* spp.) for patients requiring antifungal treatment [14, 48, 59, 60, 71]. Finally, shortly thereafter, deep-sequencing and metagenomic analysis should be performed for the re-examination of samples that do not yield the usual CF pathogens and belong to patients with non-efficient clinical outcome.

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3. Vers le concept du microbiote et mycobiote pulmonaire

Dans le contexte de la mucoviscidose (Sibley *et al.*, 2006; Filkins and O'Toole, 2015), il nous est donc apparu essentiel d'étudier l'ensemble de cette flore poly-microbienne respiratoire et d'appréhender les interactions potentielles entre les agents microbiens, de façon à améliorer la compréhension physiopathologique et la prise en charge thérapeutique de cette maladie. Depuis le début des années 2000, l'approche culture-indépendante par séquençage à haut-débit qui permet une lecture multiparallélisée de plusieurs milliers de séquences, a modifié notre approche et connaissance de l'écologie microbienne. Le terme "microbiome" a été proposé en 2001 par Joshua Lederberg (Lederberg and McCray, 2001) et est ici utilisé pour décrire la communauté écologique composée de microorganismes commensaux, symbiotiques, et/ou pathogènes qui habitent notre corps. L'utilisation des termes « microbiome », « microbiote » (microbiota en anglais) reste encore controversée dans la littérature. Dans ce manuscrit, le terme « biote » (microbiote, mycobiote) est défini comme l'ensemble des microorganismes résidant dans un site (un tissu ou organe) donné, tandis que le terme en « biome » (bactériome, mycobiome, virome) désigne l'ensemble des génomes des microorganismes (bactéries ou micromycètes ou virus et phages) composant la communauté microbienne du site donné et qui sont soumis aux pressions environnementales de ce site. Les projets américain « Human microbiome project » (HMP, http://www.hmpdacc.org/) (The NIH HMP Working Group, 2009) et européen « MetaHIT » http://www.metahit.eu/) (The Human Microbiome Project Consortium, 2013) ont été lancés pour identifier et caractériser la composition et évaluer le rôle en santé humaine des microbiomes humains de différents sites du corps, incluant le tube digestif, les voies nasales, la cavité buccale, la peau et les voies urogénitales de sujets sains et malades. La littérature sur le microbiome humain est donc en constante augmentation, grâce notamment au développement récent exponentiel des techniques moléculaires de séquençage à haut-débit.

3.1. Historique du concept du microbiote/microbiome pulmonaire

Lorsque les projets HMP et MetaHIT ont été lancés en 2008, la recherche du microbiote pulmonaire n'était pas incluse parmi celui des différents sites du corps car historiquement les poumons humains étaient considérés comme stériles selon des études basées essentiellement sur les méthodes microbiologiques conventionnelles (examens directs et cultures microbiologiques). Ce n'est qu'en 2010 en utilisant la technique de séquençage à haut-débit, que Hilty et al., (Hilty *et al.*, 2010) ont caractérisé le microbiome de brossages bronchiques

obtenues par bronchoscopie chez 3 groupes de sujets : des patients atteints de broncho-pneumopathie chronique obstructive (BPCO), des patients atteints d'asthme et des sujets sains (groupe témoins). Ces auteurs ont montré pour la première fois que l'arbre bronchique humain contenait un grand nombre de bactéries même chez les sujets sains. Dans la même année, Erb-Downward et ses collègues (Erb-Downward *et al.*, 2010) ont comparé le microbiote issu de lavage broncho-alvéolaire (LBA) de patients atteints de BPCO et celui de sujets sains et obtenu des résultats similaires. Une des limites de ces deux études était qu'il persistait un doute sur un risque de contamination par le microbiote pharyngé, et/ou celui de la cavité buccale et/ou des voies respiratoires supérieures des échantillons lors du passage du bronchoscope à travers le tractus respiratoire. Aussi dans une étude plus récente, Charlson et al. (Charlson *et al.*, 2011) ont contourné cette limite en utilisant la technique du double bronchoscope leur permettant de comparer le microbiote des deux voies respiratoires supérieures et inférieures chez six individus sains. Concernant cette technique, les voies respiratoires ont été anesthésiées avec un premier canal et le second canal maintenu stérile a été utilisé pour obtenir les brossages bronchiques protégés (BP) et LBA, évitant ainsi le plus possible la contamination. En accord avec les deux études précédentes, cette étude a également révélé un microbiote des voies respiratoires inférieures certes moins abondant que celui des voies respiratoires supérieures mais avec des OTUs bactériens non trouvés dans les voies respiratoires supérieures. Ces observations ont confirmé la présence d'un microbiote pulmonaire spécifique même chez les sujets sains ce qui a conduit à proposer le concept de microbiome et microbiote pulmonaire. L'étude du tractus respiratoire a donc été secondairement incluse dans le programme HMP. Depuis quelques années, le microbiote pulmonaire est devenu un nouveau domaine de recherche en pleine émergence qui concentre l'attention des chercheurs de différents domaines comme la microbiologie, l'écologie microbienne, la physiopathologie, la pneumologie, ou bien encore les maladies infectieuses.

Etant donné que l'accès au microbiote des voies inférieures nécessite le recueil de LBA ou BP et donc une intervention médicale non-anodine, le recueil d'expectorations représente le mode d'échantillonnage le plus largement utilisé dans les études actuelles du microbiote pulmonaire malgré la présence d'un risque théorique de contamination. L'avantage des expectorations, en effet, est la relative facilité à les obtenir. Ce sont des prélèvements non invasifs qui permettent d'effectuer plus facilement des études à grande échelle et des suivis longitudinaux de patients. Bien que cette notion reste encore controversée, la plupart des auteurs s'accordent actuellement sur une analyse du microbiote pulmonaire basée sur les expectorations.

3.2. Structure et fonction du microbiote/microbiome pulmonaire

Bien que ce microbiote soit moins abondant que celui des autres sites du corps (par exemple, on estime que la densité microbienne du microbiote pulmonaire est environ 1000 fois moins élevée que celle du microbiote bucal, et de 1 million à 1000 millions fois moindre que celle du microbiote intestinal (Adar *et al.*, 2016)), il est composé de communautés microbiennes diverses et dynamiques. La composition et la diversité de cet écosystème microbien sont uniques et caractéristiques pour chaque individu, variables selon son état de santé ou la présence de certaines maladies pulmonaires telle que l'asthme, la BPCO ou la mucoviscidose (Hilty *et al.*, 2010; Delhaes *et al.*, 2012; Goleva *et al.*, 2013; Garzoni *et al.*, 2013; Lim *et al.*, 2014; Huang and Boushey, 2015; Einarsson *et al.*, 2016; Wang *et al.*, 2016). Les modifications du microbiote pulmonaire seraient corrélées à l'évolution clinique des patients ; une dysbiose étant associée à une altération de la fonction respiratoire du patient (Delhaes *et al.*, 2012; Madan *et al.*, 2012; Zemanick *et al.*, 2013; Harrison *et al.*, 2013; Huang *et al.*, 2014; Wang *et al.*, 2016; Einarsson *et al.*, 2016). Ainsi, l'analyse NGS du microbiote pulmonaire et les nouvelles connaissances qu'elle génère suggèrent fortement que nous devons revoir notre compréhension de la pathogénèse et de la microbiologie des maladies pulmonaires chroniques en général et de la mucoviscidose en particulier. Comme le microbiome intestinal, le microbiome pulmonaire jouerait un rôle dans la régulation de la réponse immunologique. La dysbiose pulmonaire pourrait causer un déséquilibre de l'homéostasie immunologique qui interviendrait alors dans la genèse des maladies respiratoires chroniques inflammatoires (pour revue (Marsland and Gollwitzer, 2014; Dickson *et al.*, 2015b)).

Cependant, si une perte de l'abondance et de la diversité microbienne gastro-intestinales a été montrée comme associée à l'obésité, la résistance à l'insuline, la susceptibilité à l'infection et l'allergie, ou pouvant être responsable de maladies inflammatoires chroniques intestinales (maladie de Crohn), ce n'est pas le cas du microbiote pulmonaire. Les études qui ont comparé la diversité du microbiote pulmonaire des patients atteints de maladies respiratoires chroniques avec celui provenant de témoins sains nous fournissent souvent des résultats parcellaires, parfois contradictoires. Par exemple, dans la BPCO, la diversité bactérienne a été rapportée augmentée (Pragman *et al.*, 2012), ou diminuée (Erb-Downward *et al.*, 2010) et même stable (Sze *et al.*, 2012) par rapport à celle observée chez des sujets sains. Chez les patients ayant subi une transplantation pulmonaire, une diminution (Borewicz *et al.*, 2013) ou

une augmentation (Charlson *et al.*, 2012b) de la diversité bactérienne a été décrite. Ces résultats nécessitent donc des études plus approfondies avec des modélisations appropriées.

3.3. Hypothèse sur l'origine du microbiote/microbiome pulmonaire propose la modélisation d'étude du microbiote pulmonaire

Le concept de non-stérilité des poumons pose la question de l'origine du microbiote pulmonaire. Il existe plusieurs hypothèses intégrant l'impact de l'environnement, le lien avec les microbiotes des autres sites du corps (cavité buccale, voies respiratoires hautes, tube digestif…) (Gollwitzer and Marsland, 2014; Dickson *et al.*, 2015b; Adar *et al.*, 2016).

Tout d'abord, il est logique de supposer que l'inhalation de micro-organismes présents dans l'air soit une source importante du microbiote pulmonaire car on trouve dans un mètre cube d'air environ 10^4-10^6 cellules bactériennes, 10^2-10^5 particules fongiques, des millions de virus, qui circulent dans les voies respiratoires supérieures de l'Homme chaque jour (Lighthart, 2000; Denning *et al.*, 2014; Dickson *et al.*, 2015b). De plus, de récentes études ont montré que la distribution taxonomique du microbiote pulmonaire ressemble étroitement à celle des cavités buccale et nasale (Charlson *et al.*, 2011; Garzoni *et al.*, 2013; Morris *et al.*, 2013; Segal *et al.*, 2013; Venkataraman *et al.*, 2015; Bassis *et al.*, 2015). Elles suggèrent donc qu'une population majeure du microbiote pulmonaire provient des voies respiratoires supérieures et de la cavité buccale. D'autres études ont également montré un chevauchement entre la composition des populations bactériennes pulmonaire et intestinale (Rogers *et al.*, 2010; Madan *et al.*, 2012; Hoen *et al.*, 2015). Les voies respiratoires et le tube digestif pourraient donc potentiellement partager des microorganismes car ils sont liés anatomiquement via la cavité pharyngée. Ces observations pourraient s'expliquer par le fait que la voie de migration des microorganismes dans les voies respiratoires humaines repose sur des micro-aspirations de sécrétions salivaires permettant la dispersion des bactéries vers les voies respiratoires inférieures (Huxley *et al.*, 1978; Gleeson *et al.*, 1997; Dickson and Huffnagle, 2015; Dickson *et al.*, 2015b; Adar *et al.*, 2016).

D'autre part, l'utilisation des techniques moléculaires NGS a révélé que des ADN bactériens sont aussi présents dans les échantillons de liquide amniotique et placenta (DiGiulio, 2012; Aagaard *et al.*, 2014), alors que cet environnement avait toujours été présumé stérile. Cette découverte amène une autre hypothèse concernant la genèse du microbiote pulmonaire : l'exposition du fœtus au microbiote placentaire maternel pourrait contribuer à la structuration du microbiote pulmonaire du fœtus et influencer la maturation initiale des cellules

immunitaires (Marsland and Gollwitzer, 2014; Gollwitzer *et al.*, 2014; Aagaard *et al.*, 2014; Dickson *et al.*, 2015b). À ce jour, aucune étude n'a confirmé la viabilité des microorganismes du microbiote placentaire par culture ou approches transcriptomiques.

Brièvement, bien que l'origine et le développement du microbiote pulmonaire restent encore peu connus et controversés, il existe sans doute dans les poumons humains un écosystème spécifique où l'abondance et la grande diversité des différents microorganismes est variable pour chaque individu (pour revue voir Marsland and Gollwitzer, 2014; Dickson *et al.*, 2015b; Nguyen *et al.*, 2015; Nguyen and Delhaes, 2015; Adar *et al.*, 2016). Enfin, le fait que le microbiote pulmonaire soit plus semblable au microbiote de la cavité orale (Morris *et al.*, 2013) qu'à celui des autres sites suggère fortement que les voies respiratoires supérieures soient la source principale des microorganismes présents dans les poumons, par l'intermédiaire de microaspiration et de dispersion de proche en proche au niveau de la muqueuse (Charlson *et al.*, 2012a; Whiteson *et al.*, 2014a; Dickson *et al.*, 2015a).

Récemment, des auteurs ont proposé un modèle appelé le modèle « Climax/Attack » (Whiteson *et al.*, 2014b; Quinn *et al.*, 2014) pour expliquer des colonisations/infections dans la physiopathologie de la mucoviscidose et la migration des microorganismes dans les voies respiratoires. Dans ce modèle, la communauté « Climax » est considérée comme composée de populations microbiennes persistantes (par exemple *P. aeruginosa*, *S. aureus*, *Aspergillus sp.*, *Candida albicans*) qui colonisent les voies respiratoires au cours des périodes stables en s'adaptant aux réponses immunes de l'hôte et en étant plus résistantes à l'antibiothérapie à large spectre. En revanche, la communauté « Attack » est plus virulente mais transitoire, généralement composée d'agents pathogènes qui sont associés à des exacerbations pulmonaires telles que *S. pneumoniae*, *H. influenza*, rhinovirus, adenovirus, etc. et qui pourrait être plus sensible aux antibiotiques (Whiteson *et al.*, 2014b) (Figure 4). Les auteurs ont également émis l'hypothèse que les bactéries anaérobies seraient les membres de la communauté « Attack » qui pourraient être responsables du déclenchement d'exacerbation (Quinn *et al.*, 2014). Ces deux communautés peuvent coexister dans les poumons humains et contribuer ainsi à la diminution de la fonction pulmonaire.

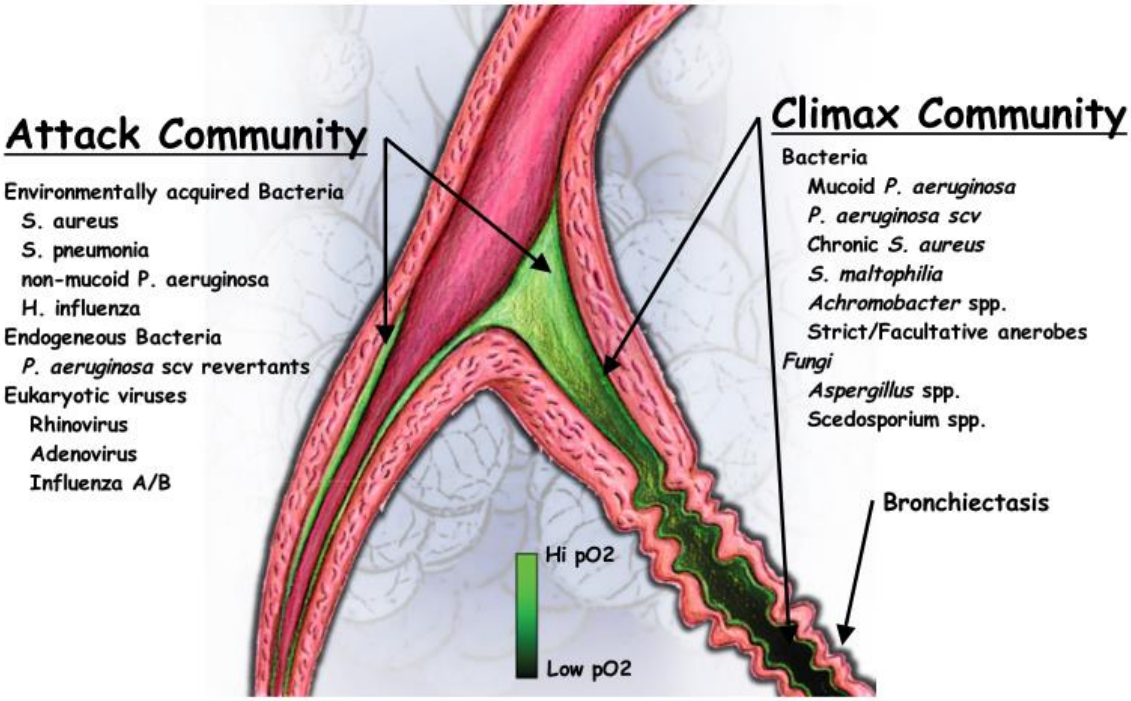

Figure 4 : Modèle des communautés Attack/Climax dans le microbiote pulmonaire chez les patients atteints de la mucoviscidose (d'après Conrad *et al.*, 2012)

D'autre part, les chercheurs ont également adapté la théorie de la biogéographie insulaire, proposée par MacArthur et Wilson dans les années soixante (MacArthur and Wilson, 1967) à l'écosystème pulmonaire (Figure 5). C'est un modèle qui permet d'expliquer la répartition et les différences entre les grands écosystèmes terrestres. Cette théorie concerne la richesse spécifique d'une île en fonction de sa taille et de son éloignement par rapport à un continent. Ainsi, une petite île qui est loin du continent aurait une faible richesse en espèces ; au contraire une grande île qui est proche du continent serait riche en espèces. Le continent est considéré comme un réservoir d'espèces, qui est la source primaire d'immigration et qui conditionne le taux d'immigration. Cette théorie indique que le nombre d'espèces sur une ile résulte d'un équilibre dynamique entre l'immigration de nouvelles espèces et l'extinction (élimination) des espèces vivant sur l'île ; le principal facteur de régulation étant alors la compétiton pour survie entre les différentes espèces de l'île. La probabilité pour qu'une espèce nouvelle immigre et s'implante sur une ile est inversement proportionnelle au nombre d'espèces présentes. Lorsque la richesse des espèces d'une île est élevée, le taux d'immigration diminue. Le taux d'immigration est zero si la richesse de l'île est équivalente à celle du continent.

Pour expliquer la biodiversité du microbiote pulmonaire, les différentes parties du tractus respiratoire humain ont été considérées comme des îles (Figure 5). Le microbiote de la cavité orale exposé à l'environnement extérieur, qui est le plus large terrain, où la richesse d'espèces est la plus élevée, qui est donc considéré comme le continent, source principale de voie de migration des microorganismes. L'abondance et la diversité du microbiote pulmonaire, loin du continent, sont donc moins élevées et moins diverses que celles des voies respiratoires supérieures. En effet, la composante du microbiote pulmonaire est déterminée par trois facteurs : l'immigration microbienne, l'élimination microbienne, et les taux de reproduction relatifs des microorganismes. Il existe plusieurs facteurs anatomiques, physiologiques, cliniques qui affectent probablement les taux d'immigration et d'élimination microbiennes du microbiote pulmonaire. Des facteurs tels que la proximité de la cavité du larynx et la charge microbienne élevée de la cavité oropharyngée augmentent probablement le taux d'immigration. A l'inverse, des facteurs tels que la toux, le tapis muco-ciliaire, ou les réponses immunes innée et adaptative, diminuent probablement le taux d'extinction/élimination. Comme les facteurs climatiques (température, humidité, précipitations, …) conditionnent l'existence des espèces sur terre, il existe également des paramètres physiologiques au niveau des poumons qui ont des effets sur les taux de croissance microbienne, tels que la concentration en oxygène, la température, le pH, la présence de métabolites…Toutes les modifications de ces facteurs ont un impact sur la richesse microbienne et l'équilibre du microbiote pulmonaire (Figure 6).

Pour conclure, bien que ce modèle n'évalue que les effets sur la richesse microbienne et non « l'evenness » avec des approches métagénomiques, il pourrait être un outil majeur pour expliquer les phénomènes physiologiques survenant dans les poumons humains et la variabilité spatiale dans le microbiote pulmonaire humain (Whiteson *et al.*, 2014a; Dickson *et al.*, 2015a).

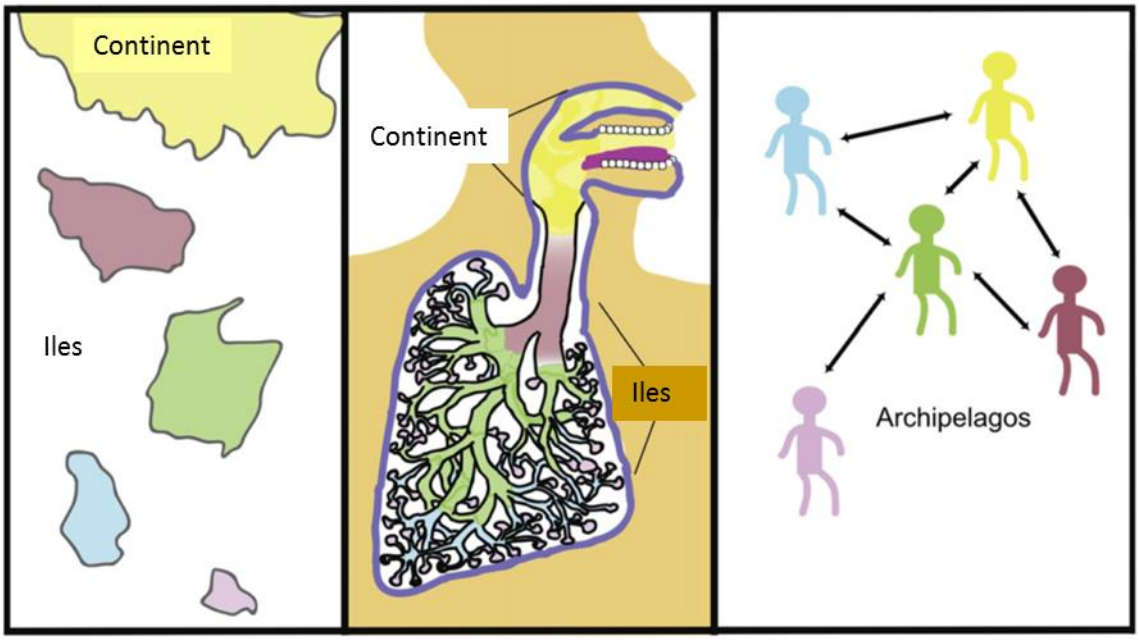

Figure 5 : La théorie de la biogéographie insulaire adaptée à l'écosystème pulmonaire humain (d'après Whiteson *et al.*, 2014a)

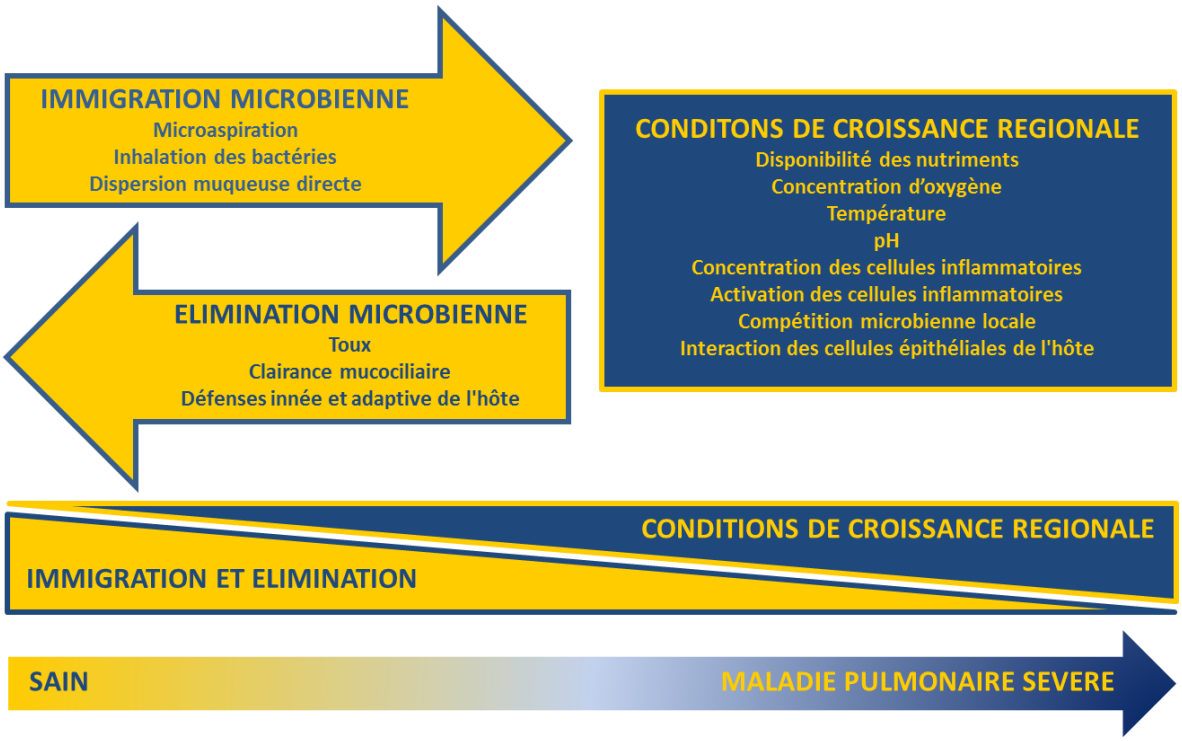

Figure 6 : Facteurs déterminants de l'écologie du microbiote pulmonaire humain (d'après Dickson and Huffnagle, 2015)

3.4. Le concept du mycobiote/mycobiome pulmonaire

Si la plupart des études sur le microbiote pulmonaire ont mis l'accent sur les bactéries et leur impact en santé respiratoire, d'autres organismes tels que les virus et les champignons sont également susceptibles de jouer un rôle important dans la physiopathologie des maladies respiratoires chroniques. Notre travail se focalise sur le mycobiote pulmonaire dont l'importance a probablement été sous-estimée, comme compartiment du microbiote mais aussi comme cofacteur d'inflammation (Marsland and Gollwitzer, 2014; Nguyen *et al.*, 2015). A ce jour, quelques études se sont intéressées au mycobiote digestif ou cutané (pour revue voir Underhill and Iliev, 2014; Marsland and Gollwitzer, 2014), très peu ont abordé la composante fongique du microbiote pulmonaire (Charlson *et al.*, 2012b; Delhaes *et al.*, 2012; van Woerden *et al.*, 2013; Willger *et al.*, 2014; Cui *et al.*, 2015). Ces études ont montré que le mycobiote pulmonaire a un impact significatif sur l'état clinique des patients porteurs de maladies respiratoires chroniques. Dans nos deux articles suivants (Article 2 et Article 3), nous avons voulu exposer le nouveau concept du mycobiome/mycobiote pulmonaire. Nous y rapportons les récentes explorations du mycobiote pulmonaire humain et discutons des synergies entre ce mycobiote et d'autres communautés microbiennes locales, ainsi que les relations avec celles d'autres microbiotes des sites du corps humain. Nous discutons également les perspectives et limites actuelles de la technique NGS appliquée au mycobiote pulmonaire. Dans ces deux travaux, j'ai réalisé la rédaction du texte et des figures sous la direction du Pr Laurence Delhaes.

Article 2 : The lung mycobiome: an emerging field of the human respiratory microbiome

Publication dans le journal *Frontiers in Microbiology*. 6:89. doi: 10.3389/fmicb.2015.00089.

Auteurs : Nguyen Do Ngoc Linh[1], Eric Viscogliosi[1], Laurence Delhaes[1,2]

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Introduction

The recent use of culture-independent microbiological techniques based on deep-sequencing has shown that the respiratory tract of healthy people are not sterile as formerly thought, but composed of a previously unappreciated complex microbial community referred as the microbiome (Charlson et al., 2010; Erb-Downward et al., 2011). In particular, two international projects were recently launched: the American Human Microbiome Project (HMP, http://www.hmpdacc.org/) and the European Metagenomics of the Human Intestinal Tract (MetaHit, http://www.metahit.eu/) (The NIH HMP Working Group et al., 2009; Huttenhower et al., 2012). They first aimed to characterize the nature, composition, and diversity of normal and pathological bacteriomes (bacterial microbiomes) at different body sites including the nasal passages, oral cavities, skin, gastrointestinal tract, and urogenital tract. The respiratory tract was secondarily included in the American program as a body site, and the lung microbiome has become a new attractive and rapidly growing field of research. However, the majority of these lung microbiome studies have focused on bacteria; the characterization of viral or fungal microbiomes - also referred as the virome and mycobiome - has not been closely investigated. It is commonly accepted that a given microbial community associated with host tissues or organs is known as the "biota", while the whole genome collection of this microbial community is mentioned as the "biome". Accordingly, "mycobiota" refers to the fungal component of a given microbial community, and the corresponding genomes are referred to as the "mycobiome" (Hooper, 2001; Iliev et al., 2012; Orgiazzi et al., 2013). While the concept of the human mycobiome has been emphasized in the past few years (Cui et al., 2013; Huffnagle and Noverr, 2013), little is known about the human mycobiome in general, and the lung mycobiome in particular as confirmed by our search in PubMed of the terms: "mycobiota", "mycobiome", "human mycobiota", "human

mycobiome", "mycobiome AND lungs", "mycobiota AND lungs", "human mycobiome AND lungs", "human microbiota and lungs", "fungal microbiome AND lungs" that appeared respectively in 190, 29, 49, 24, 272, 1, 188, 213, and 146 publications. With or without using these specific keywords, a total of 67 research publications and 29 reviews were relevant to the purpose of our review. Lung mycobiome research is clearly an "emerging world" as recently proposed (Huffnagle and Noverr, 2013), for which the growing scientific interest is supported by several reasons. First, the human respiratory tract represents the main portal of entry for numerous microorganisms primarily those occurring as airborne particles such as viruses, bacteria, or fungi. Fungal spores are representing more than 50,000 spores per cubic meter of air during the fungal season (Pashley et al., 2012; Denning et al., 2014). The corresponding microorganism characteristics, coupled with the local immune response will determine whether they will be cleared or adhere to and colonize the respiratory tract, leading to pulmonary diseases (or their infectious complications). Although the total number of fungal cells is smaller than that of the bacteria, nobody is fungus-free (Huffnagle andNoverr, 2013). Second, mycosis represents an emergent threat to public health, which has increased not only in width, but also in depth. Whereas fungal pulmonary infections represent life-threatening diseases in patients in a comparable rate to the mortality attributable to tuberculosis or malaria, it is still difficult to diagnose and treat them. Beside the most prevalent and well-known fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus*, a large number of new emerging pathogens have been described (Horré et al., 2010; Fisher et al., 2012; Marguet et al., 2012). In addition, the widespread use of antibiotics has probably influenced the balance between bacterial and fungal infections facilitating the occurrence of respiratory mycosis that are becoming resistant to antifungal drugs and difficult to treat (Chen et al., 2012; Vermeulen et al., 2013; Bousquet et al., 2014). Third, common links exist between fungi and diseases such as chronic respiratory diseases (CRD) through the immunogenic background of each individual. As part of the continuous host - pathogen - environment interaction from birth to death, fungi have been associated with asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiectasis at different levels such as fungal isolation in a sputum sample, sensitization to *A. fumigatus* or to other fungi (Fillaux et al., 2012; Knutsen et al., 2012; Speirs et al., 2012; Armstead et al., 2014; Denning et al., 2014). The presence of fungi in respiratory tract also related to worse CRD outcome (Amin et al., 2010; Chotirmall et al., 2010). Despite the fact that knowledge of the mycobiome lags behind our understanding of the bacterial microbiome, specific mycobiota have been identified in pulmonary diseases as well as in oral, digestive, and skin diseases (Ghannoum et

al., 2010; Lu et al., 2011; Charlson et al., 2012b; Delhaes et al., 2012; Knutsen et al., 2012; Harrison et al., 2013; Huang et al., 2013; Dupuy et al., 2014; Willger et al., 2014). Currently, the abundance of fungi in different body sites seems to be many degrees smaller than bacteriome as recently reported (Marsland and Gollwitzer, 2014; Underhill and Iliev, 2014). For example, gut mycobiome has been evaluated at less than 0.1% in human feces upon the MetaHIT project (Huffnagle and Noverr, 2013). Since the development of the host immune system depends on its symbiotic relationship with the microbiome, the mycobiome may act as a cofactor in the lung inflammatory response (Marsland and Gollwitzer, 2014). Therefore, it appears to be essential to understand more about respiratory mycobiome, and its potential interaction with other biomes in order to get a complete picture of the lung microbiome and to improve patient management by changing current paradigms of the disease. In this review, we summarize the recent advances in characterizing the respiratory mycobiome of patients with CRD, and discuss the mycobiome's connections with other local microbial communities, as well as the relationships with the different biomes of other body sites. These studies suggest several outlooks for this emerging field which will certainly call for a renewal of our understanding of pulmonary diseases.

Respiratory mycobiome: From proof of concept to rational data

Conventional microbial methods such as cultures of respiratory samples are useful in diagnosing fungal infections and to isolating and phenotyping microorganisms. However, they have some limitations in identifying co-infections and dynamics of polymicrobial populations. These communities consist of bacteria, fungi, and viruses all potentially contributing to infection and inflammation (Lu et al., 2011; Charlson et al., 2012a,b; Delhaes et al., 2012; Fodor et al., 2012; Filkins et al., 2012; Carmody et al., 2013; Cui et al., 2013; Harrison et al., 2013; Huang et al., 2013; Dawood et al., 2014; Fitzpatrick et al., 2014; Goffard et al., 2014; Lim et al., 2014; McCullers, 2014; Mounier et al., 2014; Purcell et al., 2014; Willger et al., 2014; Wurzel et al., 2014). In addition, the majority of fungal species are difficult to cultivate or uncultivable on usual culture media as well as new or unknown pathogens (Bittar et al., 2008; Chabé et al., 2011; Paniz Mondolfi et al., 2012). In this context, deep-sequencing methods provide an indisputable possibility to identify all microbial species (including those difficult to culture) and to generate exhaustive microbial community data. Despite recent efforts to develop high-throughput methods targeting fungal ribosomal RNA genes to characterize fungal microbiota (recently summarized by Cui et al. (2013), the published data have mainly explored the lung bacteriome. Only few studies have addressed

respiratory community and diversity of fungi (Charlson et al., 2012a,b; Delhaes et al., 2012; Harrison et al., 2013; van Woerden et al., 2013; Willger et al., 2014) or viruses and phages (Willner et al., 2011, 2012; Lim et al., 2013). But there is growing evidence to suggest the impact of viral infection on pulmonary exacerbation, and mold-related respiratory effects on CRD (Reponen et al., 2011, 2012; Hulin et al., 2013; Kieninger et al., 2013). Briefly, each lung microbiota (including mycobiota) has its own composition and evolution, which is unique and specific to each individual. It probably evolves according to the pulmonary disease and to the occurrence or not of an acute pulmonary exacerbation (Fodor et al., 2012; Carmody et al., 2013; Lim et al., 2014; Willger et al., 2014). As a consequence it is believed to play a role in the alteration of the lung function.

The lung mycobiome of healthy people are comprised various genus and species principally dominated by environment agents including *Aspergillus* species (Figures 1 and 2; Charlson et al., 2012b; vanWoerden et al., 2013; Marsland and Gollwitzer, 2014; Underhill and Iliev, 2014). For example, in the study of Charlson et al. (2012b), the most common taxa in healthy control groups are *Davidiellaceae*, *Cladosporium*, *Eurotium*, *Penicillium*. Similarly, the most abundant species of van Woerden's study (van Woerden et al., 2013) were environmental molds normally isolated from water, plant, or soil samples such as *Cladosporium cladosporioides* and *Eremothecium sinecaudum*.

The lung mycobiota of diseased subjects has been analyzed using deep-sequencing approaches for the first time in two studies conducted in 2012 (Charlson et al., 2012b; Delhaes et al., 2012). Delhaes et al. (2012) have studied the sputum samples of CF patients while Charlson et al. (2012b) investigated the bronchoalveolar lavage (BAL) and oropharyngeal wash (OW) samples of lung transplant patients suffering previously from different diseases such as CF, COPD, idiopathic pulmonary fibrosis, interstitial lung disease, and cardiovascular disease. In spite of some differences in the methodology of DNA extractions and data analysis between the two studies, the most common species or genera found in the lung mycobiome were: *C. albicans*, *Aspergillus* spp., *Penicillium*, *Cryptococcus*, *Eurotium*, in which *Candida* species dominated (Figure 1). It was reported that more than 60% of the species or genera of the fungal communities identified using 454 FLX technology (Life Sciences) were not detected by cultures (Delhaes et al., 2012). These results were recently confirmed (Willger et al., 2014). Charlson et al. (2012b) also applied the pyrosequencing method to characterize the airway microbiome in both the lower and upper respiratory tracts of lung transplant patients. It was shown that the respiratory microbial

communities in lung transplant recipients differ in structure and composition from those of healthy subjects which were found to be similar to the oral microbiome described in another recent study (Ghannoum et al., 2010). The lower respiratory tract of lung transplant subjects exhibited higher burdens of bacteria, and fungi were also detected. The richness and diversity of airway microbiota and mycobiota were markedly reduced in transplant subjects compared with control subjects, as well as in CF patients with decreased lung function and poor clinical status (Charlson et al., 2012b; Delhaes et al., 2012). In addition, macromycetes which had been found on the common trees in the forest in northern France were identified in sputum samples from several CF patients (Figures 1 and 2). This finding emphasized the significance of the exposure of the patients to the outdoor environment (Delhaes et al., 2012; vanWoerden et al., 2013).

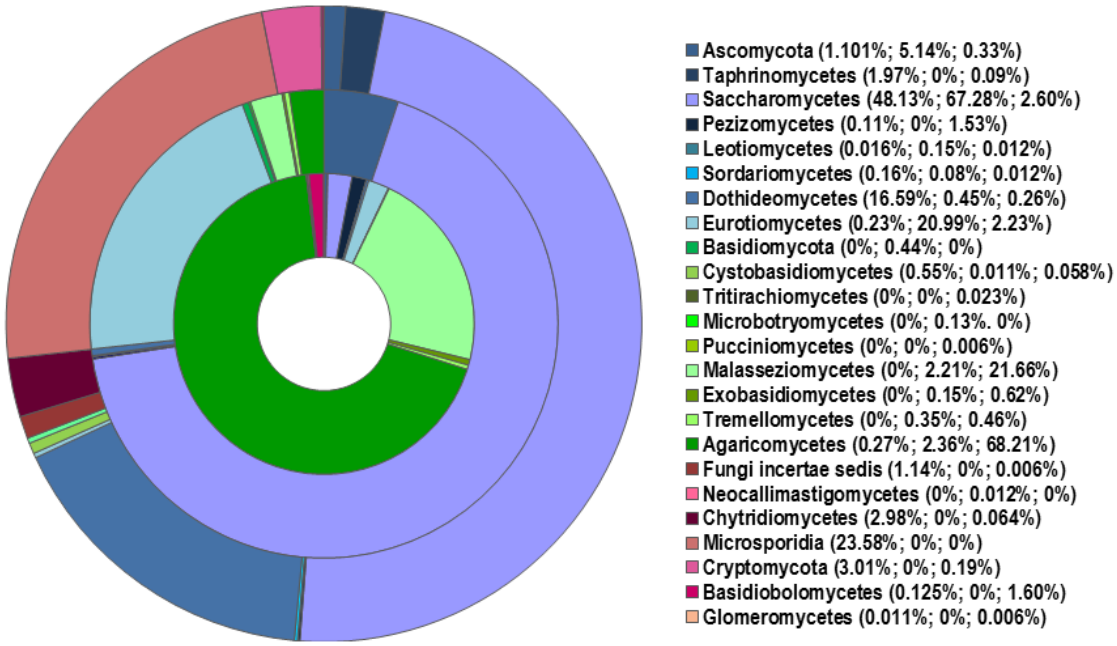

Figure 1: Distribution of fungal classes (in % of relative abundance) in the sputum of healthy individuals (outer ring) and patients with CF (middle ring) and asthma (inner ring), based on published pyrosequencing investigations (Delhaes et al., 2012; vanWoerden et al., 2013). The percentages on the legend correspond to each class identified in healthy, CF, and asthma populations (from the outer to inner rings respectively). Reads that were not identified as class level are group at phylum levels (Ascomycota, Basidiomycota). Classes less than 0.1% are not represented in the rings; the class named "Fungi incertae sedis" refers to unclassified fungi.

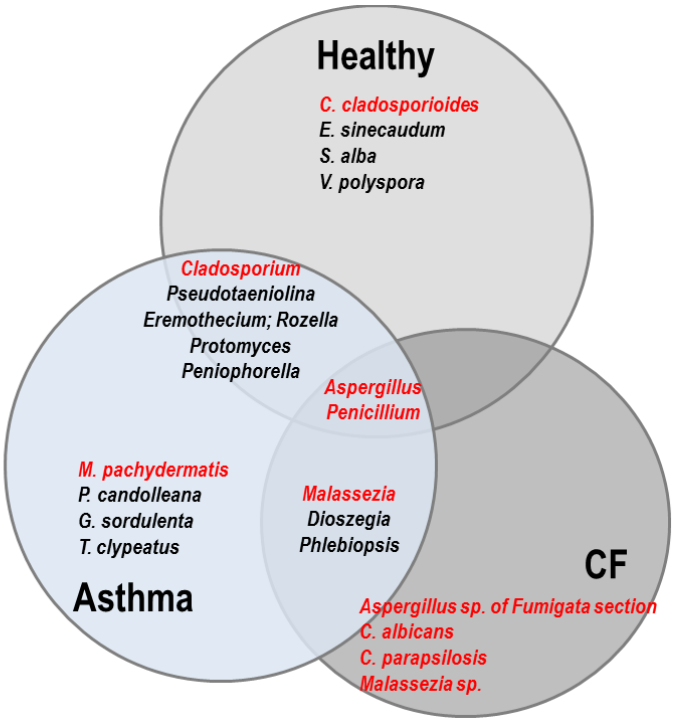

Figure 2: Venn diagram representing the comparison of the respiratory mycobiomes in healthy individuals, and patients with CF or asthma from published studies (Delhaes et al., 2012; vanWoerden et al., 2013; Willger et al., 2014). Shared genera are indicated in the overlap regions. The four most frequent species specifically isolated in each population are indicated in the non-overlap regions. Genera and species in red represent known opportunistic pathogens. In healthy people, *E. sinecaudum*, *Vanderwaltozyma polyspora*, and *Systenostrema alba* are *Saccharomycetaceae* and microsporidia isolated from soil and plants with no known clinical pathogenicity. *Cladosporium cladosporioides* has been described in cutaneous, subcutaneous, lung, and disseminated infections in immunocompromised patients. In the asthma population, *Psathyrella candolleana*, *Grifola sordulenta*, and *Termitomyces clypeatus* are environnemental Basidomycota. *Malassezia pachydermatis* is associated with atopic dermatitis. In CF patients, exclusively known opportunistic pathogens are the most frequent: *Aspergillus* species belonging to the *Fumigata* section are responsible for allergic disease (ABPA) as well as infection, while the pathogenicity of yeasts (*Candida* and *Malassezia*) is still a matter of debate in the context of CF. Among the shared fungal communities: *Eremothecium* is a filamentous fungus originally isolated from cotton; *Pseudotaeniolina* members are environmental fungi and occur only rarely in human hosts; *Rozella* is a widespread genus considered one of the earliest diverging lineages of fungi, isolated from the environment (marine); *Protomyces* is an Ascomycota phytopathogen; *Peniophorella* are soil Basidiomycota, of which few species are restricted to the northern hemisphere; *Dioszegia* are basidiomycetous yeasts found in a wide range of habitats; *Phlebiopsis* are saprotrophic fungi with a widespread distribution. *Cladosporium* species are becoming increasingly important opportunistic pathogens, especially in solid organ transplant recipients. *Malassezia* are members of the human skin flora which are associated with a wide spectrum of clinical manifestations from benign skin conditions (tinea versicolor) to fungemia in the immunocompromised host, or atopic dermatitis.

Interestingly, the *Malassezia* genus, which was detected as abundant taxa in the CF patients (Delhaes et al., 2012; Willger et al., 2014), was also found in patients with asthma but not in the control group (van Woerden et al., 2013; Figures 1 and 2). This genus, which was recently identified as the dominant taxa on human core body and arms skin (Findley et al., 2013) is known to be associated with atopic dermatitidis. In addition, pyrosequencing studies revealed the presence of *Malassezia* species in oral cavity (Dupuy et al., 2014) and in sinonasal mucosa of patients with chronic rhinosinusitis (Cleland et al., 2014). These results suggest that *Malassezia* species should have a greater interest in lung mycobiome research. Up to now, lung mycobiome exhibited significant composition differences between patients with CRD and healthy individuals according to the limited studies based on next generation sequencing (NGS) technology (Charlson et al., 2012b; Delhaes et al., 2012; van Woerden et al., 2013; Willger et al., 2014). Moreover, the authors were also able to identify the changes in the lung mycobiota over different time and/or during therapies administered and correlate these changes with the clinical context. Although the sample size was rather small (eight sputum samples from four CF patients were studied), Delhaes et al. (2012) suggested that the respiratory mycobiota plays a role in the development of CF lung pathology. *Candida* was the most commonly genus isolated in CF sputum samples, in particular *C. albicans* (Charlson et al., 2012b; Delhaes et al., 2012; Willger et al., 2014). *C. albicans* has been related to lung function decline in CF (Chotirmall et al., 2010), even if its pathogenic role remained controversial. In addition, close interactions between fungi and bacteria in the lung microbiome of CRD patients have been proposed (Delhaes et al., 2012; Marsland and Gollwitzer, 2014), in agreement with the recent ecological model of CF lungs, named the "climax-attack" community (Conrad et al., 2013). In this model, climax communities are composed of persistent bacterial and fungal populations (for example *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aspergillus* spp., *Scedosporium* spp.) which colonize the airway during the stable periods. The attack communities are more virulent, usually composed of pathogenic microorganisms which are associated with pulmonary exacerbations such as *S. pneumoniae*, *H. influenza*, *Rhinovirus*, *Adenovirus*, etc.). Both attack and climax communities can coexist in CF patients' lungs and together contribute to the lung function decline. In this model, the climax community seems to be able to avoid the patients' immune responses and/or is more resistant to broad-spectrum antibiotic therapy. In contrast, the attack communities are expected to be more susceptible to antibiotics. This model or other mathematical modeling together with metagenomic methods could help predict the effects of antimicrobial treatments on the lung microbiome, leading to a more effective use of antibiotic

and/or antifungal drugs with different treatment regimens. On the whole, these findings provide novel approaches to addressing the relationship between microbial communities and pulmonary disease. It could help to assess lung infections by favoring the development of new therapeutic approaches and models. More investigations should be carried on this field to improve our knowledge of the role of the mycobiome in the lung microbiome and in chronic diseases, such as CRD.

Relationships between the lung and gut microbiomes: Focus on the mycobiome

Despite limited research data, the concept of a relationship between the respiratory and gut microbiomes has recently been considered (Duytschaever et al., 2011; Madan et al., 2012; Huang, 2013). The human gastrointestinal tract is colonized by a complex microbial community, which has been extensively studied during the last decade. The gastrointestinal microbiome is now considered an organ with an important role in digestive metabolism, in the development and homeostasis of host inflammatory immune responses. Dysbiosis of the gut microbiome has been associated with a variety of chronic diseases, such as inflammatory bowel disease, obesity, type 2 diabetes, and asthma (Turnbaugh and Gordon, 2009; Huang, 2013; Karlsson et al., 2013). Furthermore, there is increasing evidence that the gastrointestinal mucosa is a predominant site of microbiome-host interactions and can contribute to the development of immune responses at distal mucosal sites. Due to ethical considerations, the small size of samples, and/or technical problems, studies conducted on human beings to investigate the relationships between the gut and lung microbiomes are limited. However, animal models have been developed to test the influence of the gut microbiome on the lung microbiome and immunity. Using a mouse model of cefoperazone-induced gastrointestinal microbiome disruption, Noverr et al. (2004, 2005) demonstrated that the gastrointestinal dysbiosis was characterized by increased numbers of enteric bacteria and *C. albicans*. They also proved the impact of the gastrointestinal microbiome perturbation on immune responses in the lung. Only mice with altered gastrointestinal microbiota developed airway allergic responses to intranasal administration of ovalbumin, associated with significant increases in the levels of eosinophils, mast cells, interleukin (IL)-5 and IL-13, INFγ in the lungs, and IgE in serum. The eosinophilic nature of the inflammatory response was confirmed by lung histological analysis. The allergic response was also induced by intranasally delivered conidia of A. fumigatus using the same model to perturb the gut microbiome either in Balb/c or C57BL/6 mice. These studies demonstrated that the effects of gastrointestinal microbiome disruption were independent of the host genetic background, and that the airway allergic

responses following this dysbiosis were independent of the nature of the allergen challenge but require IL-13 production in mouse models (Noverr et al., 2004, 2005). A more recent study (Barfod et al., 2013) compared the bacterial communities isolated from BAL fluids, lung tissue biopsies, fecal samples, and vaginal lavage fluids of BALB/c mice. It has shown that the lung microbiota is distinct from the cecal microbiota but overlaps with the vaginal microbiota in this animal model. Barfod et al. (2013) recommended taking into account the lung microbiome and mycobiome when studying the pathogenesis of inflammatory lung diseases. To date, only two clinical trials have been conducted in CRD populations to document the relationships between the gut microbiome and the lungs (Duytschaever et al., 2011; Madan et al., 2012). Twenty-one children with CF and 24 healthy siblings were included in a cross-sectional study (Duytschaever et al., 2011) in an effort to find any significant differences between the relative composition and temporal stability of the predominant fecal microbiome in CF patients and those in their healthy siblings, using culture and DGGE methods. Siblings were chosen as controls for patients with CF to avoid the effects of several factors that may influence the composition of the gastrointestinal microbiota, such as genetic background, age, and environmental factors. However, other factors, including antibiotic management in CF or the effect of daily diet, certainly differed since patients with CF are recommended to consume a high-fat diet to meet their energy needs. While enterobacterial counts were consistently higher in CF patients, no typical DGGE fingerprints were found from the cross-sectional study. The longitudinal study performed on two patient-sibling pairs exhibited a trend toward lower temporal stability and richness in the fecal microbiome of CF patients. Both cross-sectional and longitudinal studies provided primary evidence of a continuous state of intestinal dysbiosis in CF children compared to their siblings. Both the intrinsic characteristics of the disease (such as abnormal mucus secretions and pancreatic insufficiency) and the detrimental effects of intensive antimicrobial treatment courses most likely play key roles in this dysbiosis. The second study, using the 16 rDNA gene pyrosequencing (Madan et al., 2012), investigated the respiratory and intestinal microbiota development in infants with CF followed from birth to 21 months. In observing the dominant genera, some similar bacteria were found in both gut and respiratory microbiota, such as *Veillonella* and *Streptococcus*. Bacterial diversity increased significantly over time in both the respiratory and intestinal tracts, and in which the respiratory microbiota increased more rapidly. A significant proportion of bacteria increasing in the gut were also increasing in the respiratory tract. Furthermore, changes in diet (such as breast-feeding or introduction of solid foods) also result in modified microbiomes, suggesting an authentic link between

nutrition and the development of a microbial lung community (Madan et al., 2012). These data suggest that gut colonization patterns and nutritional factors play key roles in the development of the respiratory microbiome. Changes in the intestinal microbiome also reflect changes in the oropharyngeal microbiome (including the mycobiome component), which may directly influence the respiratory microbiome and host immune response through microaspiration (Charlson et al., 2012b). The mycobiota of the oral cavity in healthy individuals has been well characterized (Ghannoum et al., 2010; Dupuy et al., 2014). Among the 15 genera identified and composed of cultivable and non-cultivable species, Candida [isolated from 75% of all study participants (Ghannoum et al., 2010)], *Aspergillus*, *Fusarium*, and *Cryptococcus* were predominant. More recently, a high prevalence and abundance of the genus *Malassezia* was discovered by revising current practices in sequence-based taxonomy assignments (Dupuy et al., 2014). Like the oral bacterial microbiome, the oral mycobiome exhibited great variation within and between individuals that may influence the respiratory and intestinal mycobiomes in healthy individuals as well as in patients (Noverr et al., 2004, 2005; Aas et al., 2005; Ghannoum et al., 2010; Kieninger et al., 2013; Dupuy et al., 2014;Mukherjee et al., 2014). Altogether, these studies support the concept that the indigenous microbiome and mycobiome of the gastrointestinal tract have a profound influence on the development and maintenance of the respiratory microbiome, as well as lung immunity and inflammation. Further studies targeting intestinal - respiratory microbiome interactions are likely to yield important insights into the dynamics and homeostasis of microbiomes, consequently yielding a better understanding of how intestinal dysbiosis may have notable functional consequences in the pathogenesis of CRD. These findings will represent opportunities for establishing the clinical relevance of early intervention in CRD with altered dietary or frequent antibiotic therapy that may contribute to the development of dysbiosis, or probiotic strategies that might change the colonization of the gut plus lung and thereby improve patients' outcomes. As this field advances over the next several years, we anticipate that studies using larger cohorts, control groups such as siblings, multicenter designs, and longitudinal sampling will add to our knowledge of the lung microbiome and mycobiome.

Outlooks: The respiratory mycobiome or a forgotten player that deserves our attention?

Next generation sequencing technologies provide the opportunity to simultaneously analyze the microbial community (bacteria, viruses, and fungi) as a whole in an integrative research methodology without *a priori* knowledge of existing microorganisms, and consequently represent the most promising investigational strategy in the context of CRD (Figure 3). In the

last half decade, interest in the human lung bacteriome and its role in developing CRD have increased substantially. As in the intestinal microbiome, bacteria are certainly the most represented microorganisms of the respiratory microbiome, but Archaea, viruses (including phages), and Eukaryota (including fungi) also formed part of this respiratory microbiome, and may reveal clinical significances especially when dysbiosis occurs. However, little to nothing is known about the role of fungi in establishing and maintaining a healthy respiratory ecosystem, or on the other hand in facilitating pulmonary diseases. In agreement with recent published data (Findley et al., 2013; Huffnagle and Noverr, 2013), fungi may play an important role in the stability of the human microbial community, thus affecting human health and disease. Given the significantly increased rate of allergic diseases such as asthma in the westernized countries, understanding the role of the lung microbiome especially the mycobiome will be of interest in coming decades. In addition, there is evidence that the fungal exposure is associated with asthma and chronic inflammatory pulmonary diseases (Reponen et al., 2011, 2012; Hulin et al., 2013; Kieninger et al., 2013; Pringle, 2013). Fungi are also potential pathogens of the lungs while they probably act as commensals in the gastrointestinal tract, making the mycobiome analysis valuable or even higher in the respiratory tract. When considering the respiratory microbiome as a complex and continuous polymicrobial ecosystem composed of viruses, bacteria, and fungi (as a parallel from Island biogeography recently proposed by Whiteson et al. (2014)), these microorganisms may have mutual interferences. Unfortunately, their co-occurrence in the lung microbiome is poorly appreciated. To date, few studies underlined the impact of cross-kingdom synergy between bacteria and fungus in the respiratory tract and the sinonasal mucosa (Delhaes et al., 2012; Boase et al., 2013; Cleland et al., 2014). The limited understanding of fungal–bacterial interactions and their function in respiratory diseases and healthy lungs results primarily from our incomplete knowledge of the respiratory mycobiome. Overall, information obtained from oral cavity and gastrointestinal ecosystems indicates that fungi influence bacterial behavior through different interactions (i.e., positive and negative influences between and among microbiome members; Duran-Pinedo et al., 2011, 2014; Iliev et al., 2012). As in other body sites, the interactions between fungi and bacteria may occur in the lungs at physical and chemical levels. The physical interactions are mainly represented by co-occurrence or co-exclusion phenomena, while the chemical interactions include metabolic needs, quorum-sensing exchanges, and the production of antimicrobial agents. Fungi and bacteria are able to generate biofilm structures which protect bacteria and/or fungi against desiccation, antibiotic diffusion, or immune cell attacks. It results in the development of strains that are

multiresistant to antimicrobial agents and able to disseminate. Co-infection by these pathogens forming mixed communities has elevated virulence and resistance, and resilience properties that are significantly different from those of single-species communities (reviewed in Wargo and Hogan, 2006). Animal models have also shown the impact of cross-kingdom synergy between bacteria and fungi, supporting a potentially mutualistic partnership between *Candida* and *Streptococcus* (Roux et al., 2009; Diaz et al., 2012; Mason et al., 2012; Xu et al., 2014). By comparing cross-kingdom relationships associated with health and CRD using in vitro and in vivo studies, "omics" approaches will revolutionize the identification and characterization of fungal–bacterial interactions. Correlation network information already allowed authors to identify *Prevotella* species as growth co-partners of *Tannerella* sp. HOT286 and consequently to cultivate the uncultivated *Tannerella* bacteria in a co-culture system (Duran-Pinedo et al., 2011). Other authors revealed significant inter-bacteria associations at different body sites of healthy people (Faust et al., 2012). By exploring co-occurrence patterns of fungi and bacteria, Mukherjee et al. (2014) identified and explored antagonisms between *Candida* and *Pichia* yeasts in oral rinse samples. Furthermore, these bacterial–fungal interactions often have an important impact on the biology of the host metabolism and immunity as demonstrated in the gut microbiome (reviewed in Ianiro et al., 2014; Underhill and Iliev, 2014); they might also play a role in chronic pulmonary diseases and the pulmonary transplantation system, which emphasizes the need to define the respiratory mycobiome.

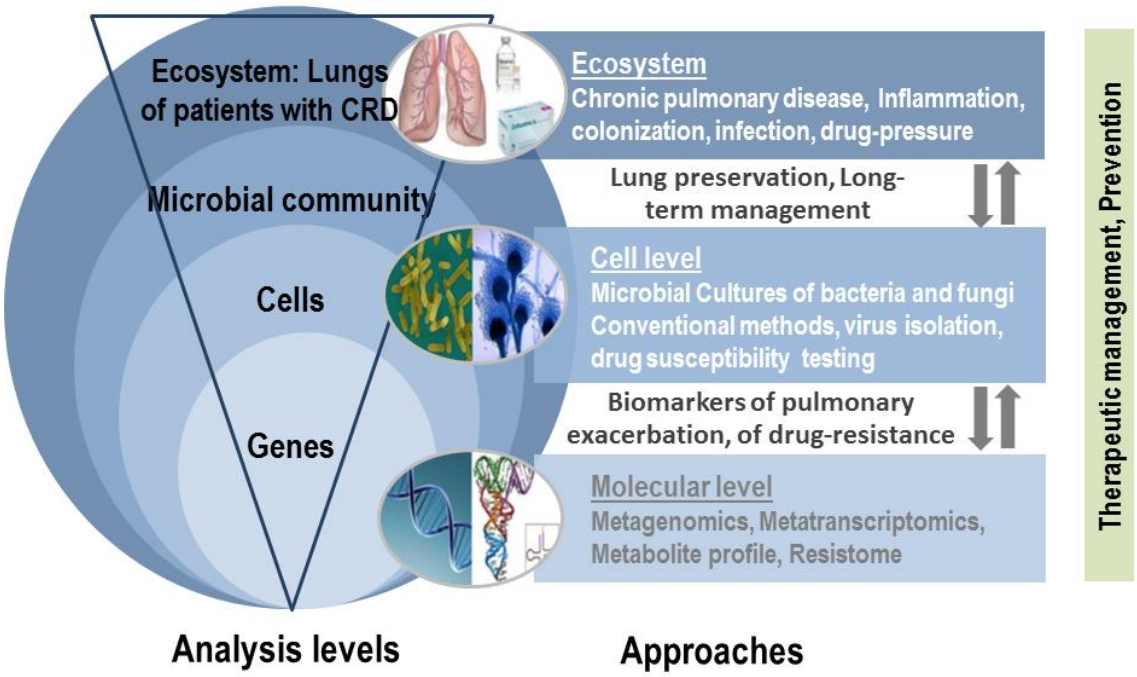

Figure 3: Integrative research based on the lung mycobiome, virome, and bacteriome.

Conclusion and future directions

For a few decades, metagenomic research has focused on the bacterial microbiome; however, clinical and experimental studies have started to highlight a role for fungi in the human microbiome in general, and in the respiratory microbiome in particular. Such a polymicrobial community has emergent properties that cannot be inferred by studying its components separately. In this respect, the "omics" approaches will be significantly helpful in identifying and deciphering candidate fungal–bacterial interactions essential to maintaining a healthy respiratory microbiome, or on the other hand to facilitate CRD. The rapid development of NGS technologies has opened up possibilities to better define in a novel way, the compositions and functions of the respiratory mycobiome and microbiome in homeostasis, during infection, and in the context of CRD. It is now essential to develop robust universal methodological strategies, and to implement large multicenter studies. In spite of recent methodological advances, the current metagenomic approaches used to study the mycobiome still have certain limitations. As proposed by Diaz et al. (2014), there are numerous pitfalls associated with the mycobiome that we need to challenge collectively. First, fungal cells are notoriously difficult to break open and might require chemical or mechanical lysis as a pre-extraction step (Chen et al., 2002; Fredricks et al., 2005; Griffiths, 2006; Plassart et al., 2012; Dupuy et al., 2014; Goldschmidt et al., 2014). Another technical point in measuring fungi using a DNA based method, is to be able to distinguish the DNA of living microorganisms and that of dead ones. Several recent studies (Nocker and Richter-Heitmann, 2010; Rogers et al., 2013; Chiao et al., 2014; Cuthbertson et al., 2014) demonstrated that a sample pre-treatment with propidium monoazide (a chemical molecule able to penetrate exclusively into cells with an intact membrane which are considered as viable cells) modified the bacterial community profiles obtained by NGS. The second challenge is to improve the taxonomic assignment quality by establishing an accurate updated fungal database. Since the internal transcribed spacer (ITS) regions of the rDNA are widely used for fungal species identification and recently formalized as universal DNA barcode markers for fungi (Delhaes et al., 2012; Schoch et al., 2012), several projects have been conducted to develop fungal databases, such as ITSDB or UNITE for QIIME released (http://qiime.org/home_static/dataFiles.html) (Diaz et al., 2014) or ISHAM ITS Database (http://its.mycologylab.org/). The later ITS database was recently generated from quality controlled ITS sequences, which represent the actual sequence variation found in each species.

Given that fungi represent the largest family of microorganisms with multiple names, another challenge is to prevent unnecessary nomenclatural flux, as recently proposed (de Hoog et al., 2014). In this context, the ISHAM ITS or UNITE databases represent a nice opportunity to create an accurate database with consensual nomenclature. Further advances in data generation and analysis are also required to minimize errors and misinterpretations, to make cross-studies and multicenter trials more feasible, and finally to link the bench and the clinic. To understand the biological function of the respiratory mycobiome in host inflammatory lung responses, its implication in CRD progression, its role in local cross-kingdom microbial interactions, as well as in the cross-talk between the intestinal and lung microbiomes, future large-scale cross-sectional and longitudinal studies must be conducted to answer these questions raised. By analogy to the microbiota signatures recently proposed for assessing responses to dietary interventions in obese individuals (Korpela et al., 2014) and for predicting future exacerbations in bronchiectasis (Rogers et al., 2014), determining both specific mycobiota and microbiota respiratory signatures could be useful for prophylactic or therapeutic management in CRD. These microbiome and mycobiome signatures might also serve as specific biomarkers preceding the clinical manifestations of disease (CRD), which should be more sensitive than routine culture methods. They might then be an indicator for timely clinical intervention and successful disease management (Lim et al., 2014). The goal is to establish both the mycobiome and microbiome in the respiratory tract over time during a respiratory superinfection or an acute pulmonary exacerbation, in order to provide key elements (signatures, dysbiosis) for early diagnosis and appropriate preventive therapy of secondary infections. Longitudinal studies will help in analyzing fungal–bacterial interactions simultaneously during the CRD progression and therapy outcome. All these additional studies are needed to generate hypotheses that in vitro and animal models will explore. By taking into account the total respiratory microbiome (that does not only consist of bacteria but also fungi, phages and viruses), these future studies will be dramatically instrumental in improving our knowledge of the pathogenesis of CRD and in developing innovative therapies.

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Article 3 : Un nouveau concept : Le mycobiome pulmonaire

(The emerging concept of lung mycobiome)

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Introduction et fondement du concept de mycobiome pulmonaire. Alors que le microbiome bactérien et son rôle en médecine sont largement étudiés, peu d'études ont porté sur la composante fongique (mycobiote et mycobiome) des communautés poly-microbiennes humaines. Quelques études se sont intéressées au mycobiote digestif ou cutané (pour revue [1, 2]); très peu ont abordé la composante fongique du microbiome pulmonaire [3-7]. Pourtant plusieurs éléments démontrent l'importance de la flore fongique dans les pathologies respiratoires chroniques telles que la BPCO, l'asthme ou la mucoviscidose :

-(i) Les spores (ou conidies) de champignons sont de petite taille (2 à 10µm), très facilement inhalée. Notre appareil respiratoire est donc exposé quotidiennement à des milliers de particules fongiques (un adulte respirant environ 15 m^3 d'air par jour contenant de 10^2 à 10^5 spores [8]).

-(ii) La relevance clinique d'une colonisation fongique, ou d'une sensibilisation (telle que l'aspergillose broncho-pulmonaire allergique ou ABPA) a été largement démontré dans la mucoviscidose et l'asthme. Jusqu'à présent, l'étude de la colonisation fongique a porté sur les espèces capables de pousser en condition standard de culture telles qu*'Aspergillus, Candida*, et à un degré moindre *Scedosporium* et *Exophiala,* ces différents agents fongiques étant susceptibles d'augmenter la mortalité et la morbidité des patients [8].

-(iii) Les traitements antibiotiques contre les infections bactériennes pourraient faciliter le développement des infections fongiques pulmonaires chez ces patients. De même, l'utilisation de traitements immunosuppresseurs (corticostéroïdes au long court ou autres) favorise le développement d'infections fongiques invasives et graves.

-(iv) Enfin, les champignons sont capables d'interagir notamment avec des bactéries (pour revue [9]). Ils peuvent aussi former des biofilms et induire une résistance aux traitements anti-infectieux.

In fine, aucun individu n'est « fungus free » [10]. Et le concept de mycobiome (incluant le mycobiome pulmonaire) représente aujourd'hui un nouveau champ de recherche en pleine expansion que les techniques de séquençage haut-débit rendent plus accessible [1, 2, 10]. Son étroite corrélation avec les pathologies pulmonaires, la réponse immune locale, ainsi que les mycobiomes des autres sites corporels commencent à être analysés [1, 2].

Du concept vers l'établissement du mycobiote pulmonaire sain et son corollaire le mycobiote dysbiotique. Aujourd'hui, le microbiote pulmonaire est considéré comme une communauté poly-microbienne composée de bactéries, de virus et phages, mais aussi de micromycètes. Cependant, la dynamique spatiale et temporelle de cette communauté fongique reste peu étudiée et connue, tout comme ses conséquences éventuelles sur la progression de la pathologie respiratoire ou la prise en charge thérapeutique [2]. A ce jour moins quelques études ont recherché et identifié le mycobiote pulmonaire du sujet sain et/ou les profils de mycobiote modifié (ou dysbiose) caractérisant un contexte pathologique spécifique (asthme, mucoviscidose, et transplantation pulmonaire). En utilisant les approches de séquençage haut-débit qui offrent la possibilité théorique d'identifier toutes les espèces fongiques présentes (y compris celles réfractaires à la culture comme *Pneumocystis jirovecii*) et de générer des données détaillées, précises et sans à priori sur cette communauté microbienne, toutes ces études ont mis en évidence une flore fongique beaucoup plus riche que celle identifiée par méthode conventionnelle (culture et/ou PCR ciblant un pathogène) [3-7].

Chez les sujets sains [1, 2, 4, 6], le mycobiote pulmonaire comporte de nombreux champignons issus de l'environnement mais aussi des *Aspergillus*, des *Cladosporium*, des *Penicillium* et beaucoup de levures (Figure 1). Sa composition est proche de celle du mycobiote buccal [1, 2, 4]. Chez les patients atteint de mucoviscidose, les mêmes genres ont été identifiées : *Candida* en particulier *Candida albicans*, *Aspergillus*, *Penicillium*, *Cryptococcus* et *Eurotium* prédominent (Figure 1) [4, 5]. Les caractéristiques écologiques de ce mycobiote (diversité et richesse des espèces fongiques) semblent corrélées à l'évolution clinique des patients : un statut clinique peu favorable étant associé à une moins grande diversité et richesse fongique [4, 5]. De façon surprenante, les espèces du genre *Malassezia* ont été isolées en grande quantité chez ces patients [5, 7]. De même, l'espèce *Malassezia pachydermatis* a été isolée uniquement chez les patients asthmatiques au cours d'une étude cas-témoins [6] (Figure 1); or *M. pachydermatis* est connue pour être responsable de troubles

allergiques (dermatite atopique). Le genre *Malassezia* est également présent au sein du mycobiote cutané (son habitat naturel), de la cavité buccale et la muqueuse naso-sinusale de patients avec sinusite chronique [1, 2].

Ces premières études, très encourageantes, montrent que le mycobiote pulmonaire diffère d'un individu à un autre, qu'il évolue dans le temps, en fonction du contexte clinique. Sur le plan descriptif, la mise en évidence des espèces du genre *Candida* et *Malassezia* est à noter. En effet, les *Candida* ont déjà été associés à une dégradation de la fonction respiratoire. Les données sur *Malassezia* (notamment sa présence dans les sphères ORL et respiratoire) laissent à penser que ce genre pourrait avoir un rôle important dans la stabilité de ces écosystèmes.

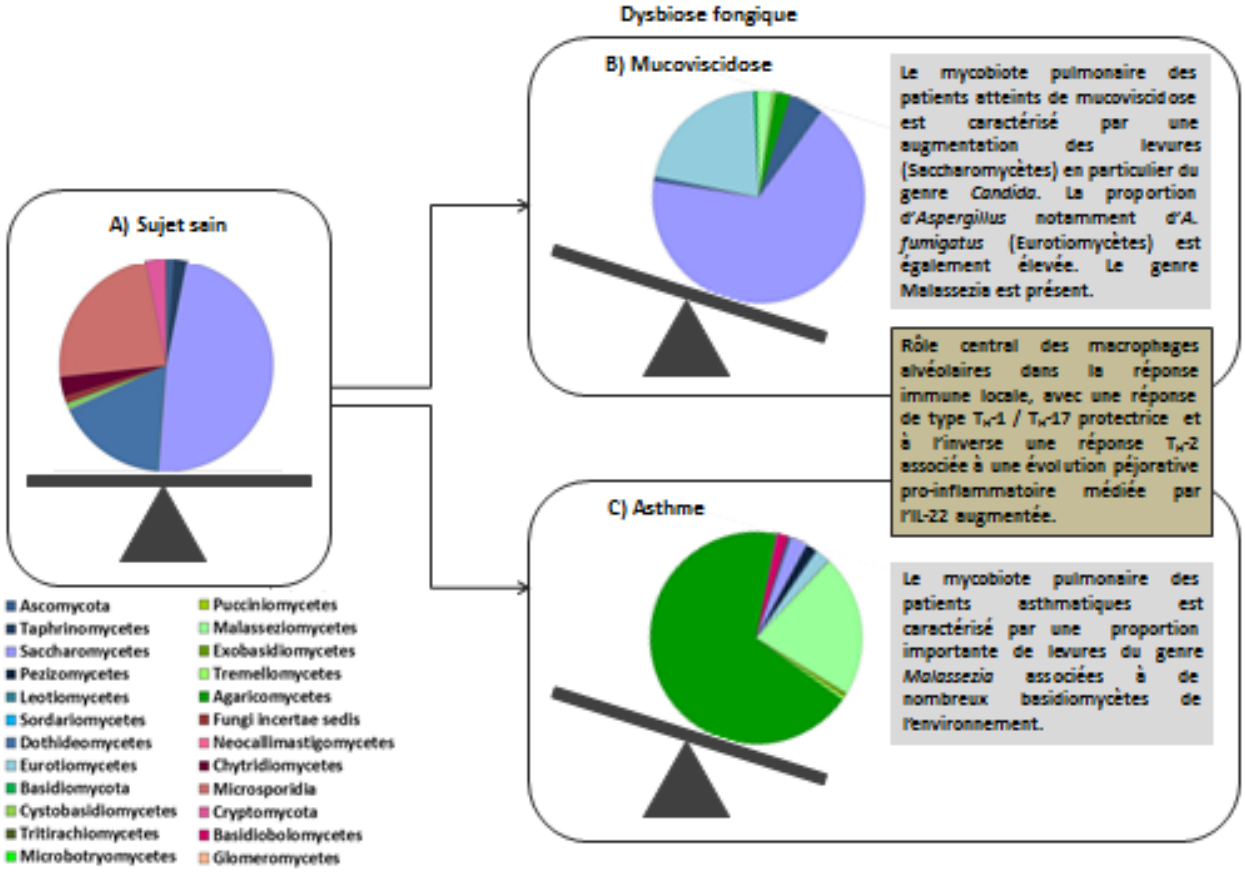

Figure 1. *Mycobiote dysbiotique au cours des pathologies respiratoires chroniques*

Perspectives : Vers une intégration du mycobiome pulmonaire dans l'étude et la prise en charge des maladies respiratoires chroniques. Même si des améliorations méthodologiques et une plus grande standardisation restent à faire, les approches de séquençage haut-débit représentent aujourd'hui une stratégie d'analyses novatrice et prometteuse tant sur le plan méthodologique que conceptuel en modifiant notre façon d'appréhender la pathologie pulmonaire.

Mieux connaitre la communauté fongique pulmonaire et sa dynamique ou écologie, la comparer avec celle de la communauté bactérienne permettra de mieux analyser les synergies potentielles entre microorganismes. De nombreuses interactions intra-, inter-espèces, et même inter-règnes (micromycètes-bactéries) ont été démontrées directement et indirectement [2, 9]. Dans ce contexte, ces approches dites en « omic » d'abord essentiellement descriptives, vont être une aide précieuse pour identifier toute interaction potentiellement clés dans le maintien du microbiome pulmonaire du sujet sain ou à l'inverse associées à une dysbiose et une pathologie respiratoire. Ces approches doivent donc désormais participer à la compréhension des différents phénomènes physiopathologiques impliqués dans les pathologies respiratoires chroniques. En intégrant la dimension écologique, elles devraient nous permettre d'appréhender différemment une altération de la fonction pulmonaire ainsi que de l'impact du traitement anti-infectieux sur l'écosystème poly-microbien respiratoire.

En conclusion, de notre biodiversité microbienne nait notre spécificité et singularité ; l'ensemble des microorganismes incluant bactérie, virus et micromycètes jouant un rôle dans le maintien d'un système respiratoire optimal. A l'inverse un déséquilibre ou dysbiose de cette flore poly-microbienne signe une évolution pathologique. De ce nouveau paradigme combinant l'étude des microorganismes qui nous colonisent aux concepts écologiques est né un enthousiasme scientifique légitime, qui a déjà commencé à révolutionner notre conception de nombreuses pathologies humaines mais qui doit maintenant prendre en compte les microorganismes non-bactériens en particulier fongiques et intégrer l'appareil pulmonaire comme un nouveau site d'étude du microbiome.

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V. RESULTATS

La mucoviscidose est une maladie chronique avec des exacerbations provoquées par des surinfections microbiennes (souvent bactériennes, parfois fongiques) pour lesquelles l'enjeu reste une prise en charge thérapeutique adaptée. En particulier, les infections pulmonaires chroniques à *P. aeruginosa* représentent une cause majeure de mortalité. Ce germe opportuniste trouve dans les poumons des patients atteints de mucoviscidose une niche qui lui permet de se développer sous forme de biofilms et de modifier l'environnement microbien local (existence de co-infections, problématique de dynamique des populations microbiennes co-présente) (Wargo and Hogan, 2006; Peleg *et al.*, 2010). De plus, certains micromycètes (comme *C. albicans* et *Aspergillus* spp) sont fréquemment isolés dans les voies respiratoires des patients après l'antibiothérapie, ce qui est un facteur de risque de survenue d'une candidémie (Burns *et al.*, 1999; Lin *et al.*, 2005).

Bien que l'existence d'une communauté pulmonaire poly-microbienne composée de bactéries, de virus et phages, mais aussi de micromycètes soit un fait avéré dans la mucoviscidose, la dynamique spatiale et temporelle de la communauté fongique reste peu étudiée et connue, tout comme ses conséquences éventuelles sur la progression de la pathologie respiratoire ou sur la prise en charge thérapeutique (Marsland and Gollwitzer, 2014).

Dans ce contexte la technique de séquençage à haut-débit représente une nouvelle approche très prometteuse dans l'analyse du microbiote pulmonaire poly-microbien très complexe chez des patients atteints de la mucoviscidose, mais elle ne permet pas de différencier les microorganismes vivants des morts. Le prétraitement des échantillons par le propidium monoazide (PMA) qui est de plus en plus utilisée pour des analyses microbiologiques et qui permet de cibler sélectivement l'ADN des cellules vivantes. Des études récentes montrent que le PMA modifie la communauté bactérienne obtenue par NGS (Nocker *et al.*, 2010; Rogers *et al.*, 2013; Vaishampayan *et al.*, 2013; Chiao *et al.*, 2014; Exterkate *et al.*, 2014; Venkateswaran *et al.*, 2014). Notre étude (Article 4) vise à déterminer si un prétraitement par le PMA modifie les mycobiote et bacteriote respiratoires déterminés par NGS chez des patients atteints de mucoviscidose, rendant la quantification des microorganismes vivants par le PMA-NGS plus relevant cliniquement.

Nous avons étudié l'impact du traitement par PMA par la technologie NGS IonTorrent sur les communautés fongique (locus ITS2) et bactérienne (régions variables V3–V5 de l'ADNr 16S)

co-présentes dans les expectorations de patients atteints de mucoviscidose, chroniquement colonisés par *P. aeruginosa* et suivis à l'hôpital de Gand.

La structure de la métacommunauté microbienne entre échantillons traités par PMA et non traités était comparable et stable puisqu'aucune différence significative entre le nombre d'OTUs et les indices de biodiversité n'était observée entre les deux groupes. Au niveau du bactériote, aucune différence significative n'a été identifiée entre les deux groupes pour les populations totales et abondantes mais il existait une différence significative des indices de Simpson pour la population intermédiaire (OTUs<1%, p=0.029) et une tendance à la différence pour l'indice de Shannon (p=0.057). Nous n'avons pas trouvé de différence significative entre les 2 groupes au niveau du mycobiote. Les résultats de beta-diversité confirment ceux de la diversité alpha.

Notre étude a permis de montrer que le prétraitement par PMA modifiait légérement l'abondance relative en OTUs et la biodiversité des mycobiote et bacteriote respiratoires, notamment au niveau des populations bactériennes intermédiaires. Néanmoins, un tel protocole étant couteux et chronophage, nous discutons de son intérêt potentiel dans une approche multidisciplinaire du suivi des patients atteints de mucoviscidose lors de réponses inadaptées au traitement antimicrobien. D'autres études basées sur l'utilisation du PMA seront nécessaires pour mieux documenter nos connaissances en "omic".

Ma contribution dans cette étude a été de participer à l'analyse des données et à la rédaction complète de l'article.

Article 4 : Effects of propidium monoazide (PMA) treatment on mycobiome and bacteriome analysis of cystic fibrosis airways during exacerbation

Publication acceptée avec les révisions mineures dans le journal *PLoS ONE.* Septembre 2016

Auteurs : Linh Do Ngoc NGUYEN[1], Pieter DESCHAGHT[2], Sophie MERLIN[3,4], Alexandre LOYWICK[3,4], Christophe AUDEBERT[3,4], Sabine VAN DAELE[5], Eric VISCOGLIOSI[1], Mario VANEECHOUTTE[2], Laurence DELHAES[1,6,*#]

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<u>Article 4 :</u> Effects of propidium monoazide (PMA) treatment on mycobiome and bacteriome analysis of cystic fibrosis airways during exacerbation

Publication acceptée avec les révisions mineures dans le journal *PLoS One*. Septembre 2016

Short title: PMA impact on cystic fibrosis mycobiome and bacteriome analysis

Auteurs: Linh Do Ngoc NGUYEN[1], Pieter DESCHAGHT[2], Sophie MERLIN[3,4], Alexandre LOYWICK[3,4], Christophe AUDEBERT[3,4], Sabine VAN DAELE[5], Eric VISCOGLIOSI[1], Mario VANEECHOUTTE[2], Laurence DELHAES[1,6,*#]

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Abstract

Propidium monoazide **(**PMA)-pretreatment has increasingly been applied to remove the bias from dead or damaged cell artefacts, which could impact the microbiota analysis by high-throughput sequencing. Our study aimed to determine whether a PMA-pretreatment coupled with high-throughput sequencing analysis provides a different picture of the airway mycobiome and bacteriome.

We compared deep-sequencing data of mycobiota and microbiota of 15 sputum samples from 5 cystic fibrosis (CF) patients with and without prior PMA-treatment of the DNA-extracts. PMA-pretreatment had no significant effect on the entire and abundant bacterial community (genera expressed as operational taxonomic units (OTUs) with a relative abundance greater than or equal to 1%), but caused a significant difference in the intermediate community (less than 1%) when analyzing the alpha biodiversity Simpson index (p=0.03). Regarding PMA impact on the airway mycobiota evaluated for the first time here; no significant differences in alpha diversity indexes between PMA-treated and untreated samples were observed. Regarding beta diversity analysis, the intermediate communities also differed more dramatically than the total and abundant ones when studying both mycobiome and bacteriome. Our results showed that only the intermediate (or low abundance) population diversity is impacted by PMA-treatment, and therefore that abundant taxa are mostly viable during acute exacerbation in CF. Given such a cumbersome protocol (PMA-pretreatment coupled with high-throughput sequencing), we discuss its potential interest within the follow-up of CF patients. Further studies using PMA-pretreatment are warranted to improve our "omic" knowledge of the CF airways.

Keywords: Cystic fibrosis, mycobiome, mycobiota, microbiome, lung microbiota, lung mycobiota, propidium monoazide (PMA), high-throughput sequencing, NGS.

Introduction

Cystic fibrosis (CF) is associated with severe lung damage because of reduced mucociliary clearance and subsequent polymicrobial infections [1]. Approximately 90% of CF patients suffer from lung destruction, promoted by pathogens such as *Pseudomonas aeruginosa*. Consequently, antibiotic treatment represents a keystone of CF therapy, preventing chronic infection and reducing exacerbation rates and alteration of pulmonary function.

Until now, strategies to manage CF lung disease principally consist of routine microbiology (including microbiological culture of sputum samples) and appropriate antibiotic treatment [1,2]. While these conventional methods identify viable and abundant pathogens, they could not detect uncultivable or difficult-to-cultivate microorganisms. The recent use of culture-independent methods based on high-throughput sequencing (HTS) has provided a more complete view of the CF lung bacterial microbiome and its evolution during respiratory alteration [3–7]. However, the current use of HTS does not differentiate the DNA of living microorganisms and that of dead microorganisms in sputum samples, which might be essential in the clinical context of exacerbation. Sample pretreatment with propidium monoazide (PMA) might facilitate viable microorganism detection, as recently proposed [6].

PMA is a chemical compound that selectively penetrates into cells with damaged membrane (dead cells), intercalates covalently into their DNA and inhibits PCR amplification of these dead microorganisms. It has been combined with different molecular methods to remove the biais from dead or damaged cell artefacts in microbial samples [6,8–17]. Exclusively several studies have shown that the PMA cross-linking method leads to changes in the bacterial communities using HTS [6,11–15].

The aim of the present study was to evaluate whether PMA-pretreatment associated with HTS analysis is able to provide a more realistic profile of the viable fungal and bacterial communities in the CF airways, and consequently reflect more closely the clinical outcome of the patients. We compared HTS data of pro- and eukaryotic microbiota obtained with and without PMA-pretreatment of DNA extracts from sputum samples, in order to assess the impact of PMA-pretreatment when quantifying the respiratory mycobiome and bacteriome of CF patients.

Materials and methods

Ethics agreement. Ethics approval for this study was provided by the Ethics Committee of Ghent University Hospital, Belgium (project nr. 2007/503). Study was performed in accordance with approved national and international guidelines; written informed consent was obtained from all the patients > 18 years or from the parents and children older than 12 years, as previously reported [16,17].

Patients and sampling. Fifteen sputum samples were prospectively collected from 5 CF patients (3 homozygous and 2 heterozygous carriers of F508del-CFTR) followed at Ghent Hospital for acute pulmonary exacerbation. Patients aged from 15 to 34 years old and were chronically colonized with *P. aeruginosa* [1] and received the same antimicrobial treatment: Tazocin® (Piperacillin/Tazobactam 4000mg/500mg) by intravenous administration during 15 days. Sputum samples were collected as previously reported [16,17].

PMA-pretreatment. The PMA-pretreatment and DNA extraction were conducted at the laboratory for Bacteriology Research of Ghent University as previously reported [16,17]. Briefly, each sputum sample was transferred into 2 wells of a 24-well plate and 10 µl of PMA (final concentration: 50 mM) (Biotium, Hayward, CA) was added to a first 190 µl sample aliquot (for PMA-qPCR) and 10 µl saline buffer was added to the other sample aliquot. After 30 min incubation in the dark on a shaker, the samples were exposed to a 500 W halogen light source for 10 min at a distance of 20 cm. During exposure, the 24-well plate was kept on ice to avoid overheating of the samples.

Culture-based quantification of *P. aeruginosa* [16,17]. Homogenized sputum samples were diluted serially tenfold in physiological saline. Each dilution (25µl) was inoculated in triplicate on cetrimide agar plates. The *P. aeruginosa* load was determined after 72 h of incubation at 37 °C in ambient atmosphere.

Quantitative PCR targeting either *P. aeruginosa or A. fumigatus.* After DNA extraction using the easyMAG Nuclisens DNA extractor (bioMérieux, Marcy l'Etoile, France), qPCR was carried out on the Light-Cycler480 (Roche, Basel, Switzerland) as previously described [16,17]. The reaction mixture contained 5 µl of Probes Master kit, 0.5 µM of each primer (*P. aeruginosa* – oprL-gene forward: ACC CGA ACG CAG GCT ATG, reverse: CAG GTC GGA GCT GTC GTA CTC), 0.1 µM of hydrolysis probe 6FAM-5'AGAAGGTGGTGATCGCACGCAGA3'-BlackBerry Quencher) and 2.5 µl of DNA-extract, for a final volume of 10 µl. PCR annealing temperature was 55 °C. A standard tenfold dilution series was prepared using a quantified DNA of *P. aeruginosa* strain PA14 for which the genome number was estimated and translated into CFU (clony-forming unit). This

standard dilution series was used in qPCR analysis to construct a standard curve, which enabled to calculate the number of cells (in CFU) in the samples on the basis of the obtained Ct-values.

The qPCR targeting *A. fumigatus* were performed for both PMA-treated and untreated samples by real-time PCR performed with a 5-µl DNA volume on a LightCycler instrument (Roche, Meylan, France), as previously described [18].

Metagenomic library preparation, sequencing and phylogenetic assignation. The hypervariable V3–V5 regions of 16S-rRNA gene were amplified using the primers: For16S_519, CAGCMGCCGCGGTAATAC and the reverse primer Rev16S_926, CCGTCAATTCMTTTGAGTTT. The ITS2 loci were amplified using the forward primer GATGAAGAACGYAGYRAA, and reverse primer RBTTTCTTTTCCTCCGCT [19].

Indexed amplicon libraries were clonally amplified with Ion PGM™ Template OT2 400 Kit and the Ion OneTouch™ ES Instrument (Ion Torrent), and sequenced through PGM, Ion Torrent (Life Technologies).

Raw data analysis was performed using a home-made pipeline composed of open-source softwares (Mothur [20], Esprit-tree [21], biome format [22]), databanks and home-made Perl/python scripts, which were all implemented in Galaxy [23]. Briefly, the first step corresponded to a preprocessing step producing a curated and filtered collection of reads using Mothur tools [20]. All reads shorter than 150 bases were removed. The remaining sequences were trimmed to remove the erroneous homopolymers generated by the Ion Torrent PGM sequencer, with a maximum limit for homopolymer length set to 20. Once this filtering was applied, duplicated sequences were grouped to save computing time during the alignment and clustering steps. The phylogenetic analysis was based on 16S rRNA gene classification from RDP, Silva and GreenGene databases [24] and ITS2 gene classification from DBScreen database [25]. Sequences with alignments less than 100 bases were filtered out.

Statistical analyses. Data were considered as paired PMA-treated and untreated samples to study PMA impact in the ecological characterization of a given sample. According to published data [26–28], fungal and bacterial communities of each sample were divided into sub-populations as follows: Abundant taxa were defined as genera with a relative OTU abundance greater than or equal to 1% and intermediate taxa as having a relative abundance of less than 1%.

Alpha diversity indexes (richness, Shannon-Wiener and Simpson's indexes) were compared using Wilcoxon test. Beta diversity and OTU analysis were accessed using ANOSIM (analysis of similarity) method, PCA (principal component analysis), and the Bray-Curtis

similarity index. To estimate similarity and changes in community composition of paired samples, we applied a hierarchical cluster analysis based on the relative abundance of taxa in the samples using Bray-Curtis similarity and a dendrogram inferred with the unweighted pair-group average algorithms. The clustering robustness was accessed by bootstrapping (10,000replicates). The p values were corrected using the Bonferroni correction for multiple comparisons. Numerical variables were described as means and standard deviations or 95% confidence interval. P-values of less than 0.05 were considered as significant. All statistical analyses were performed using PAST software version 3.05 [29].

Results

Overall effect of PMA on the polymicrobial community in CF sputum samples. The mean abundance of *P. aeruginosa* cells in PMA-untreated samples using qPCR (total DNA, 7.44 ± 9.21 x 10^7 cells/ml) was significantly higher than that of PMA-treated samples (5.17 ± 6.50 x 10^7 viable bacteria/ml, p= 0,00006 using pairwise t-test) and that of cultures (2.91 ± 3.98 x 10^7 cells/ml), whereas *P. aeruginosa* abundances estimated by qPCR in PMA-treated and culture samples were not statistically different (Fig 1). For each pair of samples, qPCR results showed a regular positive difference between the two conditions (S1 Fig), in agreement with the expected PMA effect.

Regarding HTS raw data (ENA registered http://www.ebi.ac.uk/ena/data/view/PRJEB14967, and S1 Table), the average total number of reads (both fungal plus bacterial reads) of PMA-treated samples was slightly higher than that of untreated ones (respectively 176,135 ± 136,953 and 168,434 ± 178,433 reads). The mean length of sequences from PMA-treated samples was shorter than that of untreated samples (respectively 323 ± 22 and 328 ± 16 bp) without any statistical significance.

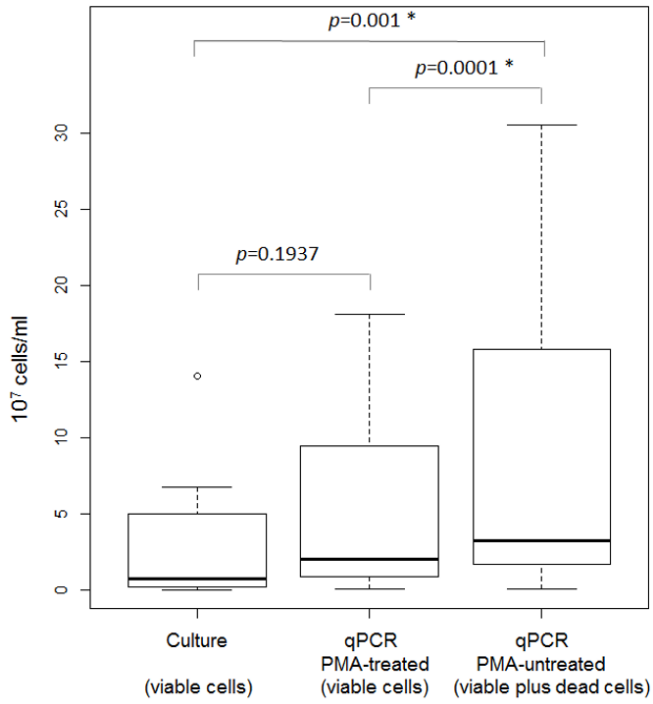
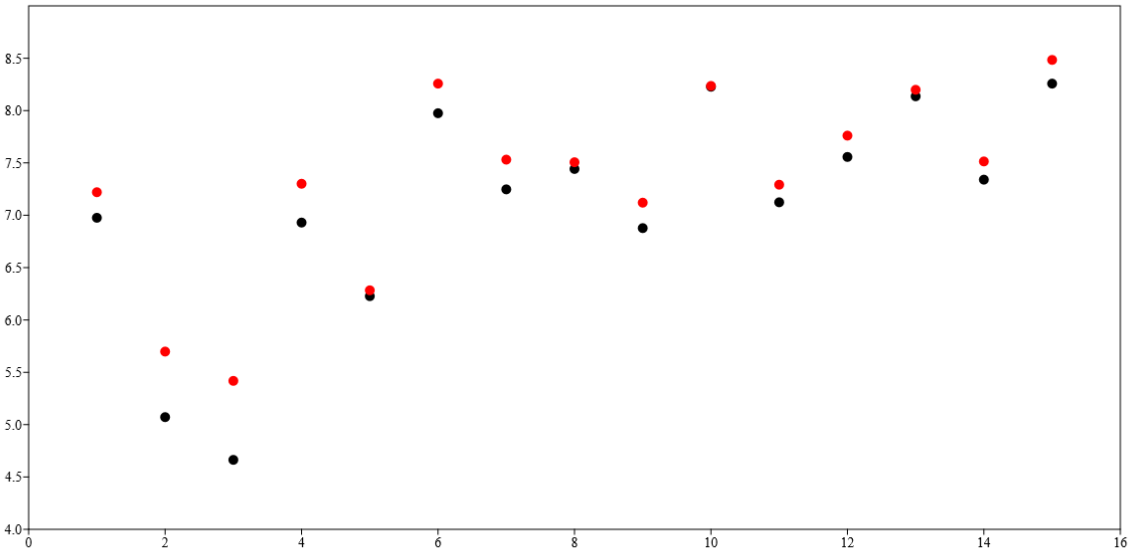


Fig 1. Comparison of the enumeration of *P. aeruginosa* cells between cultures and qPCRs of PMA-treated and untreated sputum samples.



S1 Fig. Individual comparison of *P. aeruginosa* abundances estimated by qPCR in 638 PMA-treated (●) and untreated (●) samples.

For each sputum sample, we characterized the composition and diversity of fungal and bacterial microbiota. One and 5 pairs of samples were excluded from the bacteriome and mycobiome analysis respectively since corresponding rarefaction curves and/or numbers of reads were inadequate to allow biodiversity comparison. Therefore, among the 15 paired samples, only 14 were analyzed at the bacterial NGS level and 10 at the mycological NGS level. The 14 paired samples (n=28) exhibited a total of 763,802 bacterial reads corresponding to 114 bacterial genera, and the 10 paired samples (n=20) exhibited 655,284 fungal reads corresponding to 90 fungal genera (Figs 2 and 3). As the patient status regarding *Aspergillus* colonization was not expressly known, we retrospectively assessed for *A. fumigatus* occurrence in sputum DNA extracts using qPCR in order to validate the results of ITS2 deep sequencing-based method [18]. Only sputum samples from patient "G172" exhibited positive PCR results at the limit of detection threshold (40.1 ± 2.1 Ct) [18,30] .

The number of bacterial sequences in the samples ranged from 1,560 to 55,495 reads with an average of 27279 ± 12673 (n=28). The dominant genera co-presenting with *Pseudomonas* (34.9%) were *Streptococcus* (27.4%), *Azomonas* (13.8%), *Veillonella* (6.6%), *Prevotella* (3.8%), *Porphyromonas* (3.5%), *Achromobacter* (2.0%), *Fusobacterium* (1.3%) and *Sneathia* (1.1%) (Fig 3A). The mean number of fungal sequences was 32764 ± 39977 (n=20). Dominant fungal genera were *Candida* (70.5%), genus belonging to Saccharomycetes (13.0%), and *Nakaseomyces* (11.3%) (Fig 3B).

The overall relative abundance of *Pseudomonas* genus increased from 27.8% in untreated samples to 41.4% in PMA-treated ones (Fig 3A) while the species *P. aeruginosa* was decreased after PMA-treatment, which may be linked to the antibiotic regimen (designed to kill *P. aeruginosa* cells but not all the cells of genus *Pseudomonas*). *Veillonella* also increased from 4.3% to 8.8%, and *Porphyromonas* from 2.1% to 4.8%. By contrast, a number of genera decreased in PMA-pretreated samples: in particular, the relative abundance of *Streptococcus* decreased from 29.3% to 25.5%, and that of *Azomonas* from 21.6% to 6.6%. The majority of these bacteria are described as susceptible to piperacillin/tazobactam.

Regarding mycobiota overall data, the relative abundance of *Candida* and *Nakaseomyces* decreased from 71.5% to 69.7%, and from 16.7% to 6.9% respectively (Fig 3B).

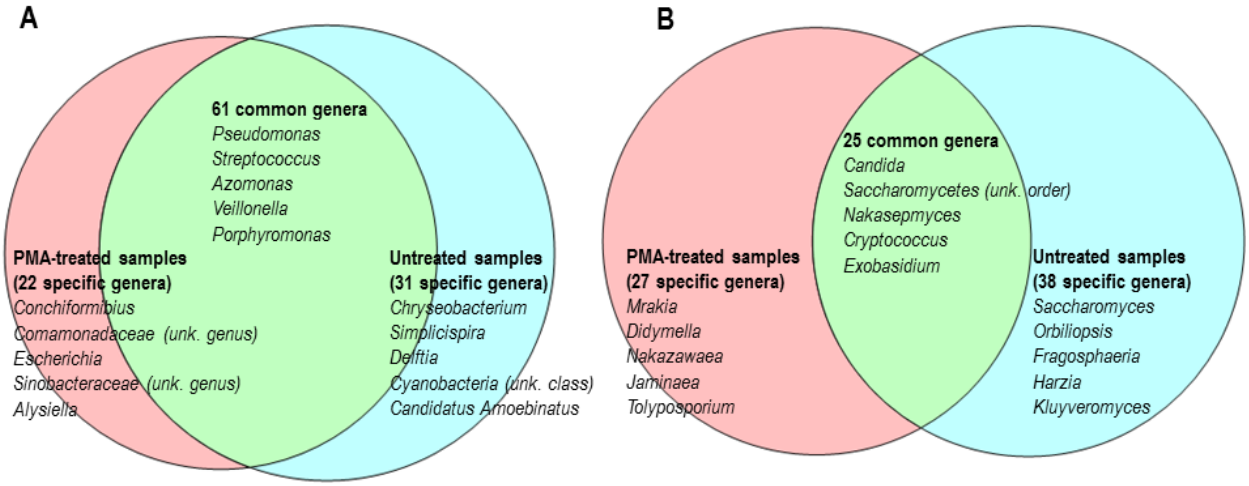


Fig 2. Venn diagram representing number of shared and specific bacterial (A) and fungal (B) genera between PMA-treated and untreated samples. In each case, the first 5 most prevalent genera are listed.

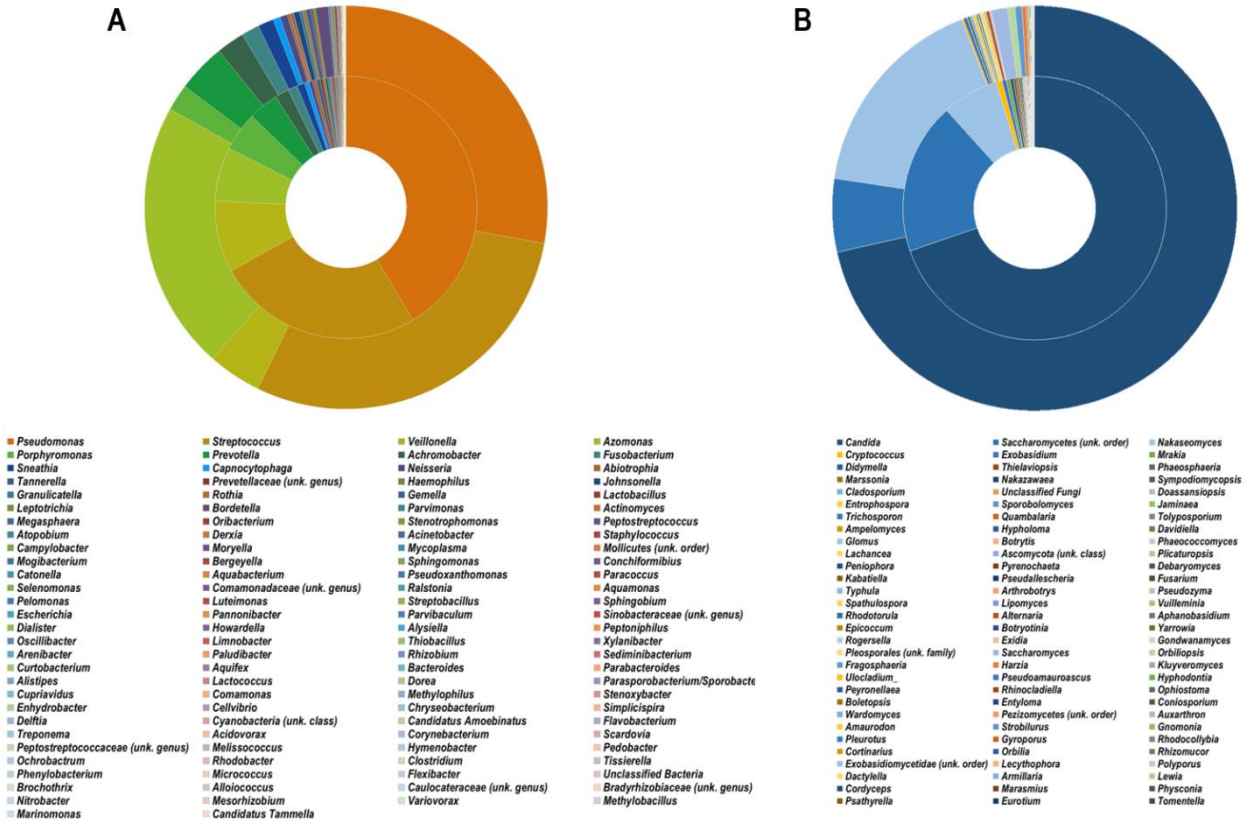


Fig 3. Global distribution of bacterial (A) and fungal (B) genera between PMA-treated (inner ring) and untreated groups (outer ring). All genera are ordered in descending relative abundance.

Impact of PMA on biodiversity and similarity among fungal and bacterial communities. PMA-treated and untreated groups shared 61 bacterial and 25 fungal genera (mostly referred as dominant genera). While a majority of known bacterial and fungal pathogens were shared by both types of samples (Fig 2), the bacterial or fungal genera referred as intermediate populations mainly belonged to the unshared populations (Figs 4A and 5A). For each sample pair, HTS results were highly congruent between the two conditions (with and without PMA-pretreatment) excepted for samples G014-III, G176-III, and G088-II (Figs 4 and 5). The genus numbers of both bacterial and fungal unshared populations were slightly higher in untreated samples than in PMA-treated ones, as confirmed by means of richness and biodiversity indexes (Table 1). More interestingly, we identified a significant difference in the intermediate bacterial population based on Simpson index ($p=0.03$) and a trend toward significance based on Shannon index ($p=0.06$) (Table 1). No significant differences in alpha diversity indexes of mycobiota data were observed (Table 1).

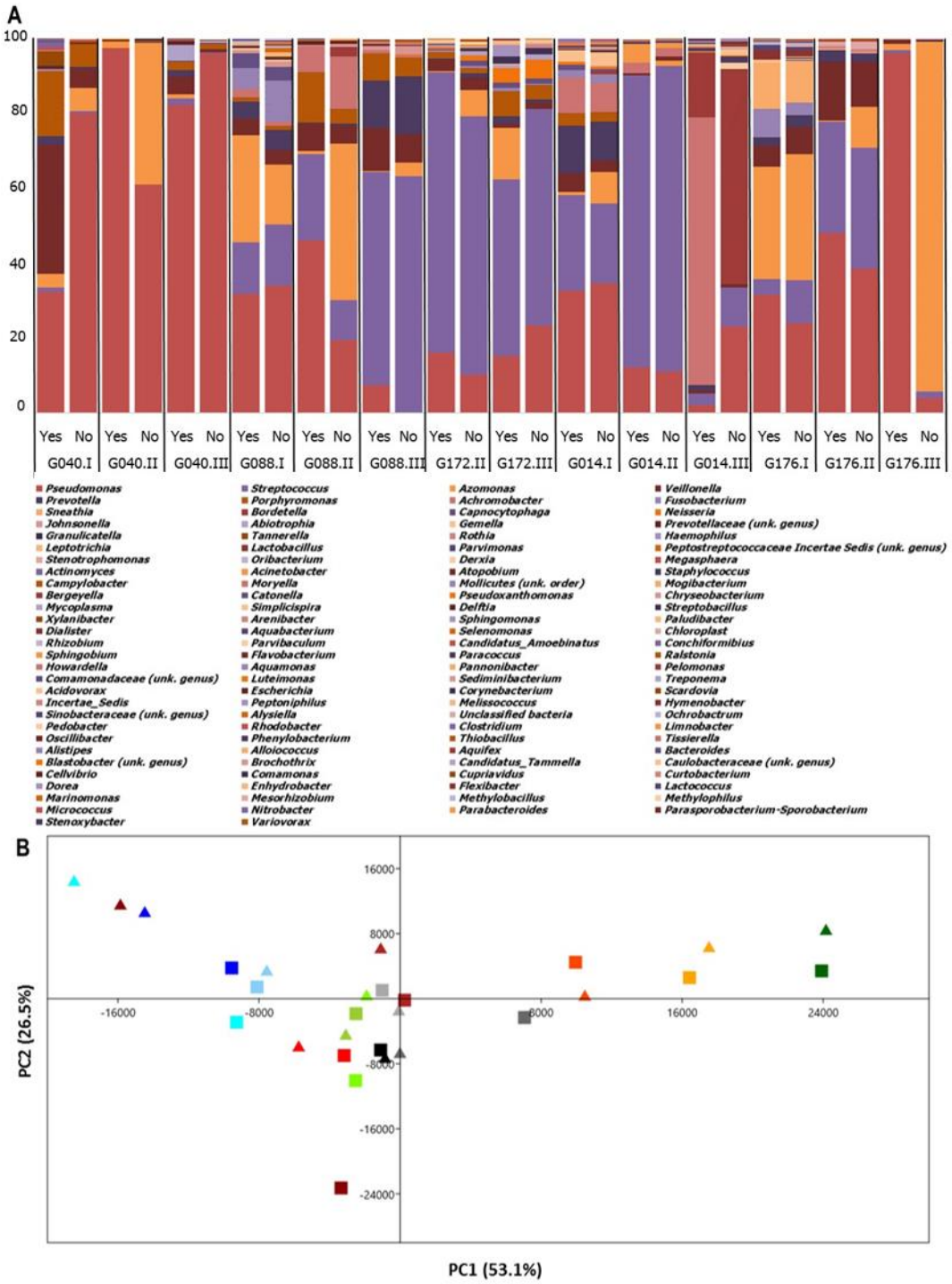


Fig 4. Effect of PMA on the composition of bacteriome of each sample. Fig 4A: Relative abundance of bacterial genera of each sample ("yes" or "no" indicates the samples with or without PMA-pretreatment). Fig 4B: PCA plot of the first two components of bacteriome of samples with and without PMA-pretreatment. Each marker represents treatment conditions (filled triangle symbols PMA-treated samples, filled square symbols untreated samples). Each color represents a given sputum sample (G040.I: light blue; G040.II: aqua; G040.III: dark blue; G088.I: yellow green; G088.II: chartreuse; G088.III: dark green; G172.II: orange; G172.III: dark orange; G014.I: grey; G014.II: dark grey; G014.III: black; G176.I: red; G176.II: firebrick; G176.III: dark red).

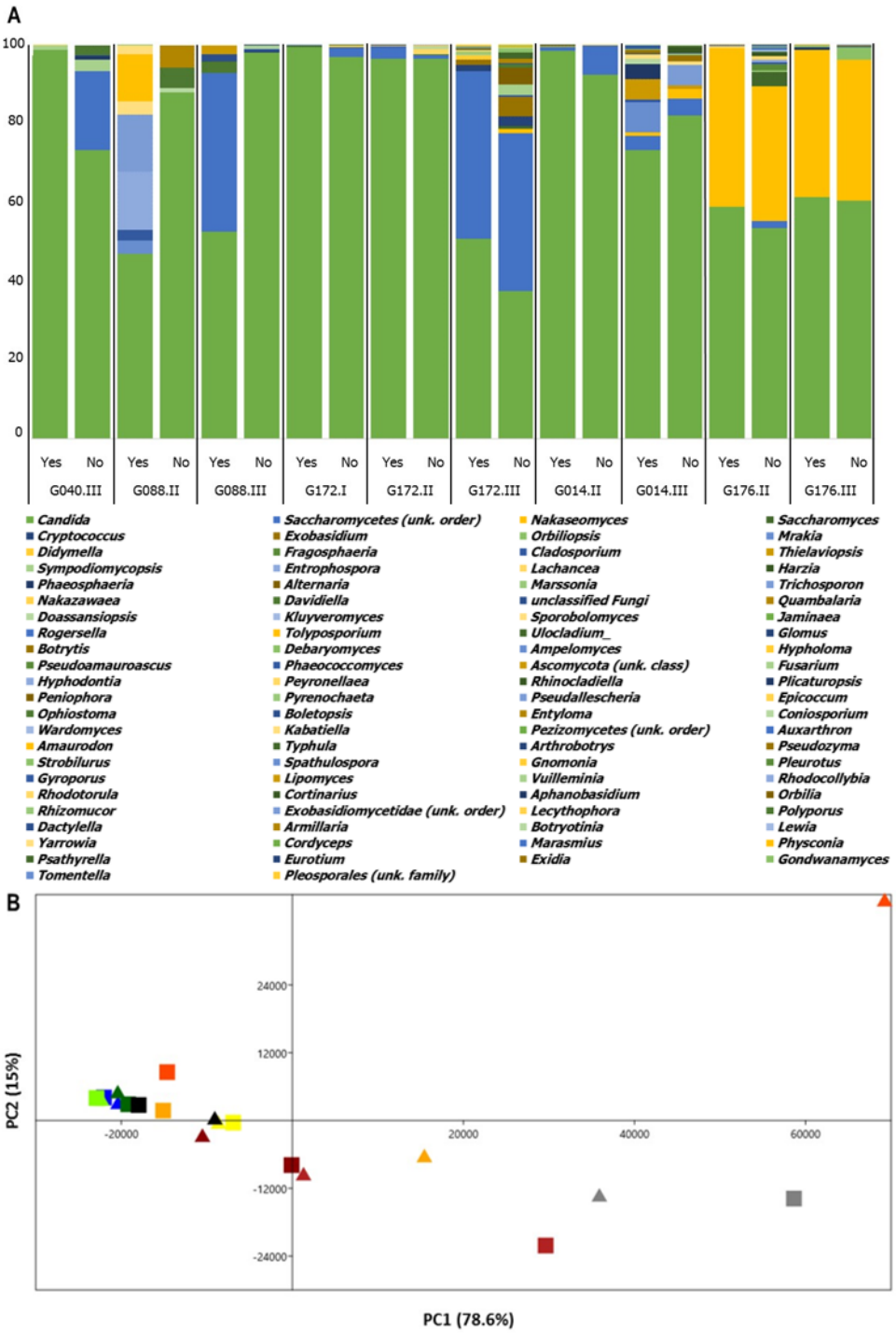


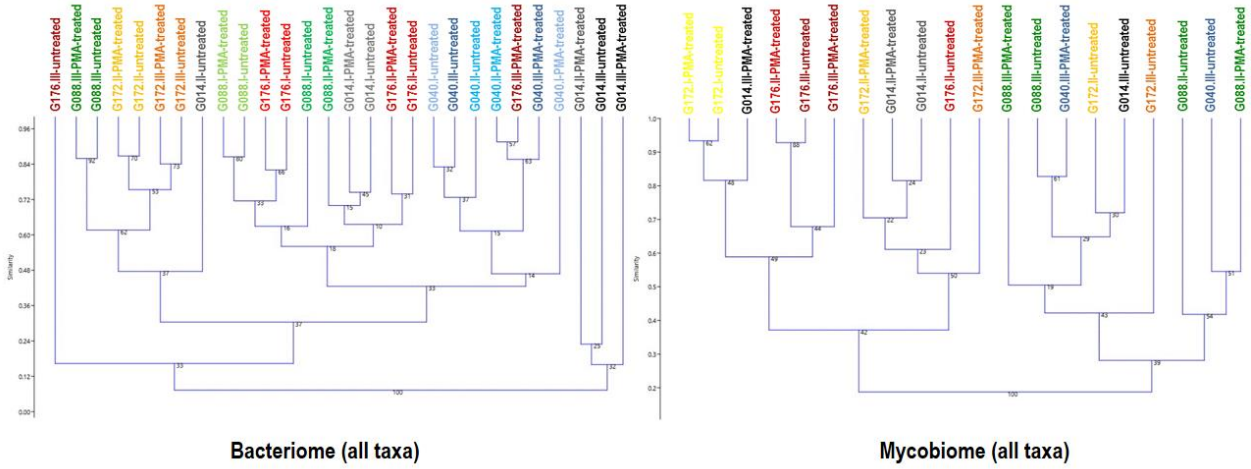
Fig 5. Effect of PMA on the composition of mycobiome of each sample. Fig 5A: Relative abundance of fungal genera of each sample ("yes" or "no" indicates the samples with or without PMA-pretreatment). Fig 5B: PCA plot of the first two components of mycobiome of samples with and without PMA-pretreatment. Each marker represents treatment conditions (filled triangle symbols PMA-treated samples, filled square symbols untreated samples). Each color represents a given sputum sample (G040.III: dark blue; G088.II: chartreuse; G088.III: dark green; G172.I: yellow; G172.II: orange; G172.III: dark orange; G014.II: dark grey; G014.III: black; G176.II: firebrick; G176.III: dark red).

Table 1. Abundance and α-diversity comparison of bacteriome and mycobiome between PMA-pretreated and untreated samples

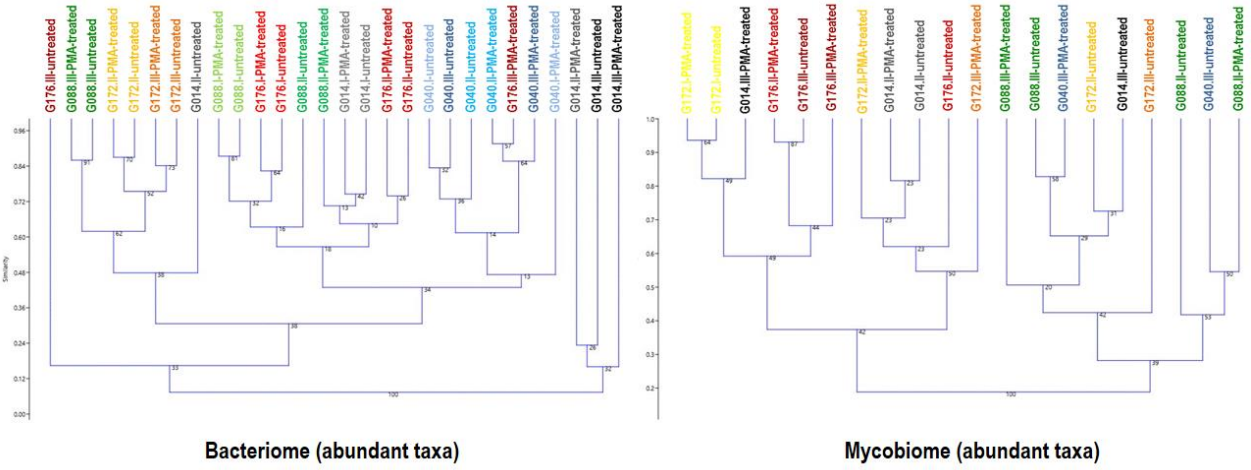
Paired sputum samples	All taxa			Abundant taxa (≥ 1%)			Intermediate taxa (<1%)		
	*PMA-treated	Un-treated	$^{\mu}p$	PMA-treated	Un-treated	*p*	PMA-treated	Un-treated	*p*
Bacteriome analysis (Number of paired samples =14) :									
**Total number of reads	28280 ± 14356	26278 ± 11194	*0.8*	27617 ± 13955	25591 ± 10873	*0.8*	663 ± 538	686 ± 490	*0.9*
Richness	20 ± 8	22 ± 11	*0.3*	6 ± 3	6 ± 3	*1*	13 ± 7	16 ± 9	*0.3*
Shannon index	1.22 ± 0.6	1.23 ± 0.6	*0.6*	1,098 ± 0.58	1.091 ± 0.56	*0.7*	1.82 ± 0.57	1.99 ± 0.6	*0.06*
Simpson index	0,53 ± 0,26	0,54 ± 0,24	*0,9*	0.51 ± 0.26	0.51 ± 0.25	*0.7*	0.752 ± 0.2	0.791 ± 0.14	***0.03***
Mycobiome analysis (Nunber of paired samples =10) :									
Total number of reads	36618 ± 44508	28910 ± 36879	*0.9*	36286 ± 43812	28637 ± 36386	*0.9*	332 ± 763	273 ± 741	*0.5*
Richness	9 ± 6	12 ± 7	*0.9*	6 ± 4	8 ± 4	*0.2*	3 ± 3	4 ± 5	*0.7*
Shannon index	0.65 ± 0.54	0.65 ± 0.5	*0.9*	0.62 ± 0.53	0.61 ± 0.48	*0.8*	0.57 ± 0.59	0.35 ± 0.3	*0.8*
Simpson index	0.34 ± 0.27	0.3 ± 0.24	*0.9*	0.33 ± 0.27	0.29 ± 0.24	0.9	0.31 ± 0.29	0.35 ± 0.3	0.7

*PMA, propidium monoazide; $^{\mu}$***p***: p value (significant p value is bolded); **Total number of reads and all diversity index expressed as mean and standard deviation.

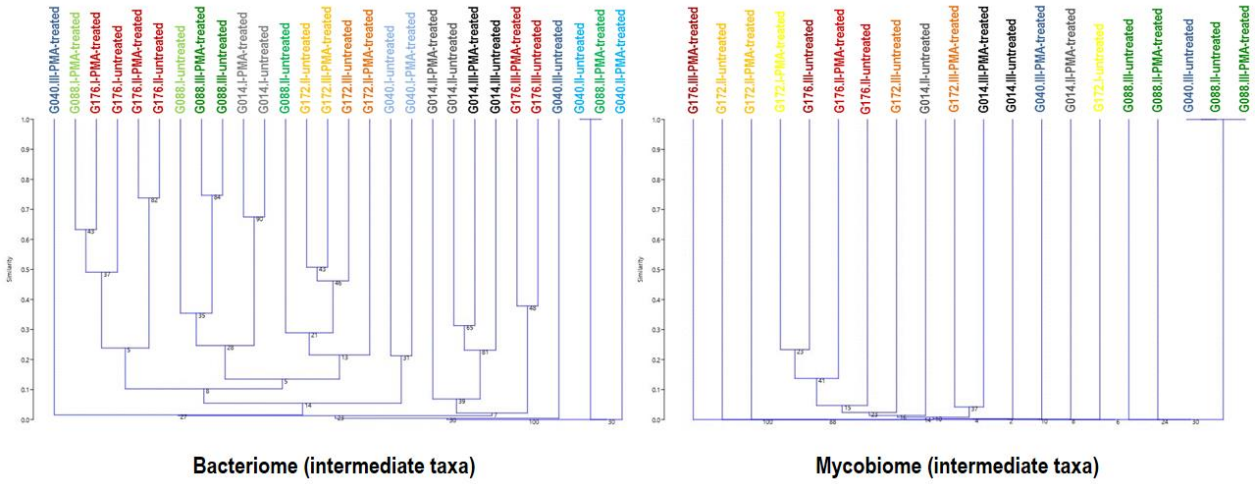
Based on Bray-Curtis similarity calculation, the clustering of the entire communities (S2 Fig) was similar to the grouping of the abundant populations (S3 Fig) for both bacterial and fungal microbiotas. Only 2 pairs of samples still exhibited such a similarity for their bacteriome when the intermediate population was analyzed (S4 Fig).



S2 Fig. Dendrograms representing the similarity between the composition of bacteriome and mycobiome from individual samples in all taxa. Clustering is based on Bray Curtis similarity distance matrix (bootstrap 10000 replicates). Bootstrap values (in percentages) are given at the nodes.



S3 Fig. Dendrograms representing the similarity between the composition of bacteriome and mycobiome from individual samples in abundant taxa (≥1%). Clustering is based on Bray Curtis similarity distance matrix (bootstrap 10000 replicates). Bootstrap values (in percentages) are given at the nodes.



S4 Fig. Dendrograms representing the similarity between the composition of bacteriome and mycobiome from individual samples in intermediate taxa (<1%). Clustering is based on Bray Curtis similarity distance matrix (bootstrap 10000 replicates). Bootstrap values (in percentages) are given at the node.

PCA and outcome of the ANOSIM of the OTU data showed similar results: the paired samples (squares versus triangle in Figs 4B, 5B) still clustered relatively close to each other. The main bacteria explaining the variance for the first axis in the PCA plots were from OTUs of *Prevotella*, and of *Pseudomonas*, *Streptococcus*, *Veillonella* for the second axis (Fig 4B). The principal fungi explaining the variance for the first axis were from OTUs of genera belonging to *Candida*, *Saccharomycetes*, and *Nakaseomyces*. The main loadings of the second axis were determined by those of *Saccharomycetes* (unknown order and genus) (Fig 5B). ANOSIM method confirmed these findings, showing no significant differences between the PMA-treated sample data and the untreated ones (Table 2). These beta-diversity results are consistent with those of the alpha-diversity indexes.

Table 2. *p-values* and *r-values* of similarity analysis (one-way ANOSIM) of the bacterial and fungal OTU data

OTU data	Population and sub-populations	**p*-values	***r*-values
Bacteriome	Global population	0.52	-0.011
	Abundant population	0.52	-0.010
	Intermediate population	0.97	-0.066
Mycobiome	Global population	0.84	-0.062
	Abundant population	0.84	-0.062
	Intermediate population	0.56	-0.009

**p- values* indicate the significant differences between PMA-treated and untreated groups;
***r-values* indicate dissimilarity between groups as follow: the higher the *r-value* is, the more dissimilar the compared groups are.

Discussion

Chronic pulmonary colonization with recurrent infective exacerbations, caused by intercurrent bacterial, viral and/or fungal infections, produces an irreversible decline in lung function and early death in CF patients [1,7]. Although a diversity of bacterial species can be isolated from the CF airways, *P. aeruginosa* is the most common CF pathogen able to develop chronic infections with acute exacerbations [1,31,32]. Once established, chronic *P. aeruginosa* infections are difficult to treat with antibiotics and the pathogen is virtually never eradicated due to biofilm formation [31]. Furthermore, *P. aeruginosa* biofilm may interact with fungi, displaying an increase in mutability in mixed biofilms [33,34]. *P. aeruginosa* consortium is also modified according to a Climax/Attack model when exacerbation occurred [3,4]. In this model, fermentative anaerobes are hypothesized to be the core members of the Attack Community and responsible for exacerbation [4]. In this context, analyzing microbial community of CF respiratory tract by using HTS coupled with PMA-pretreatment may facilitate the detection of both viable bacteria (including obligate anaerobes) and fungi potentially involved in the exacerbation, without underestimating or overestimating DNA quantifications and microbial community compositions.

While PMA-pretreatment has been combined with various molecular techniques and successfully applied for discriminating between living and dead cells in microbiology [6,8,10–17,35], it has been combined to pyrosequencing in only few studies to analyze the bacterial community of environmental and human samples [6,11–15]. Given the importance of detecting the whole viable members at metacommunity level, we studied both respiratory mycobiome and bacteriome of CF patients colonized with *P. aeruginosa* during exacerbation using PMA-pretreatment combined with HTS. Since the mycobiome in general and the lung mycobiome in particular have not been fully investigated [36], this study provides the first opportunity to focus on the PMA-pretreatment effect on this understudied eukaryotic community and its co-presence with the bacteriome in human airways during acute pulmonary exacerbation.

Apart from *Pseudomonas* identified as dominant bacterial genus (in agreement with our selection criterion of patients chronically colonized with *P. aeruginosa*), our results (Fig 3A) confirmed the core taxa of bacteria in CF respiratory microbiome previously described (for review: [5]). The 9 most abundant genera contributed 94.4% of the total number of sequences while the rest of more than a hundred genera contributed only a small proportion of the total OTU number (Fig 4A). Several bacterial species detected in the CF respiratory microbiome here were also considered as the most frequent oral commensal bacteria such as *Streptococcus*, *Prevotella*, *Porphyromonas*, *Rothia*, *Tannerella*, *Fusobacterium*,… This result confirmed the close

relation between the respiratory and buccal microbiotas due to microaspirations of salivary and potentially explained by the neutral dispersion model recently proposed [37–39]. In addition regarding the co-presence of *Pseudomonas* and oral commensal bacteria, there is recent evidences showing that the oral commensal streptococci could modulate the growth of *Pseudomonas* in CF disease condition [40].

Similar to bacteriome results, 3 fungal genera accounted for 94.7% of the total number of fungal OTUs. Among them, *Candida* genus (including *Candida albicans* (52.3%)) was the dominant genus of the CF respiratory mycobiome in our study (Fig 3B, 5A). *C. albicans* has been frequently isolated with high prevalence in CF sputa, even if its role in pathogenesis and clinical CF evolution is still matter of debate (for review: [36,41]). There is also evidence showing the co-presence of *C. albicans* and *P. aeruginosa* in patients' respiratory tract as an opportunistic damaging association [33]. The remaining fungal genera represented a limited number of OTUs, and confirmed the core taxa of lung mycobiome previously described [25,42-44]. Similar to bacteriome analysis, the high abundance of *Candida* and *Saccharomycetes* observed here might also refer to the overlap with the buccal mycobiota in agreement with published data [37–39]. Interestingly, our study identified an important proportion of *Nakaseomyces delphensis* (11.3%) in 7 of total 20 samples from 3 out of the 5 patients. This cultivable non-pathogenic yeast is closely related to *Candida glabrata* [45]. To our knowledge, it has never been detected in human respiratory tract. These results underline the large exposure of lungs to environmental microorganisms that may play a role in chronic respiratory diseases such as CF, and highlight the importance of analyzing the whole respiratory microbiota, which could be a reservoir of diverse microorganisms yet to be identified or yet to be classified as pathogens in CF (such as *C. albicans*).

As expected, PMA-pretreatment reduced significantly the amplifiable amount of *P. aeruginosa* DNA and the number of viable cells (Fig 1), in agreement with patient treatment and published data [6,17]. This result is also compatible with the ability of *P. aeruginosa* to form biofilms that contain a higher proportion of dead cells, and are observed in lungs of CF patients chronically colonized with *P. aeruginosa* as our patient population [6,17,35]. As OTU numbers were slightly higher (Table 1) and the mean length of sequencing reads was shorter in PMA-treated samples than in untreated ones, we could refer to some limitations of the PMA-treatment technique recently described [13,46,47]. Various factors affecting the influence of PMA treatment on the results of both qPCR and HTS (type of samples, PMA concentration, PMA incubation time, light source and exposure time, length of target gene, pH) have been proposed [13,46,47]. We basically followed the steps that were in agreement with an optimal effect of PMA-pretreatment. The

estimated numbers of viable cells in our samples were more than 10^7 cells/ml, in agreement with the cutoff of 10^5 cells/ml recently proposed to get the most adequate effect of PMA-treatment [35]. Furthermore, the mean length (>300 bp) of sequencing reads from both PMA-treated and untreated samples was long enough to show an accurate detection of viable cells [13]. The use of multiple viability filters (sample treatment with DNase/Proteinase K or metabolic and enzyme activity estimations) has also been suggested [11]. As the present study has some limitations such as a modest sample size (15 samples from CF patients with acute pulmonary exacerbation, without a control group composed of CF patients clinically stable) or the absence of mycology culture records that have limited the data analysis, further studies are now warranted to fully evaluate the efficiency of PMA coupled with HTS.

Besides, the "rare" or "intermediate" population has recently become an emerging concept in ecology, which has been particularly studied in sea-water samples [27,28,48,49]. Despite different cutoffs used to differentiate the minority taxa ("rare" to "intermediate" taxa), all these studies have shown that minority populations vary more than majority ones and that minority taxa play a crucial role in the ecosystem stability [27,28,48,49]. We observed a significant difference in the diversity of intermediate bacterial populations between the two groups of samples **(**Table 1**)**. This result is in agreement with Rogers et al.'s study which also evaluated the effect of PMA treatment on *P. aeruginosa* abundance through qPCR and on the diversity of the lung bacteriome from CF patients who were judged to be clinically stable at the sampling time [6]. While Rogers et al. found that PMA-treatment resulted in an increase in community evenness driven by an increase in diversity of rare community members, we identified a decrease in community diversity of intermediate population. Similar to this study, and despite the two different types of CF populations, our results suggest that there was no significant difference in the entire bacterial community but PMA-pretreatment could significantly influence the apparent composition of "satellite" taxon groups analyzed by HTS. Several other studies using HTS compared the bacterial profiles of PMA-treated and untreated environmental samples and showed significant changes in the bacteriome structure [11–14]. Whereas Vaishampayan and colleagues [12] demonstrated a difference in the bacteriome structure of environmental samples with and without PMA-pretreatment using PCA, we found both PMA-treated and untreated samples situated virtually next to each other (Fig 4B), in agreement with Exterkate and colleagues' results, studying oral cavity samples [14].

Regarding the mycobiome, we could not establish any significant difference of abundance or biodiversity between PMA-treated and untreated samples (Table 1). Despite some changes in the mycobiota compositions (Fig 5A and S2 Fig), no significant difference in both abundant and

intermediate populations was observed. To the best of our knowledge, this is the first study focusing on the effect of PMA with regard to airway mycobiome characterization by HTS, which limits the comparison and discussion of our findings. However, PMA-treatment has been recently coupled with HTS to investigate the fungal burden of room dust samples [15]. The authors found remarkable differences between the PMA-treated and untreated samples, and indicated the presence of a large proportion of pathogenic fungal genera such as *Aspergillus*, including *A. fumigatus* which represents the most frequent pathogenic mold isolated in CF respiratory samples [41]. We identified *Candida*, *Malassezia*, *Alternaria*, and *Fusarium*, but OTUs belonging to *Aspergillus* genus were not isolated. This result could be explained by some bias in DNA extraction or amplification. As we were able to amplify DNA from other phylogenetically close filamentous genera such as *Fusarium*, DNA extraction method should be sufficient to breakdown fungal cell walls. The primers we used were shown to be highly efficient for amplification of diverse fungal species including those of the Ascomycota, Basidiomycota and non-Dikarya [19]. This suggests that *A. fumigatus* was indeed not present in our samples, as confirmed with the *Aspergillus* qPCR results.

Considering the co-evolution and co–exclusion of core taxa, our results suggest that PMA-pretreatment explains better the physiopathology of these core taxa and their interactions in the CF lung environment. We report a proportion of *Pseudomonas* increasing from 27.8% to 41.4% of total reads in the PMA-treated group, in agreement with Rogers et al.'s results [6]. In parallel, OTUs of *Streptococcus*, *Azomonas*, and *Candida* genera decreased (Fig 3). These microbial proportions modified between the PMA-treated and untreated groups may reflect the behaviors of microbial communities within CF lungs: *(i)* Decrease in pH, low concentration in oxygen, high level of mucus, and bioavailability of metabolites such as alanine and lactate represent optimal conditions for *P. aeruginosa* growth, especially as biofilm consortium that PMA-treatment put prominently by differentiating viable to dead cells (for review: [39]. *(ii)* By providing an appropriate competitive niche for *Pseudomonas* genus, other genera such as *Streptococcus* and *Azomonas* may lose their survival advantages in CF lungs, a hypothesis in agreement with Filkins et al.'s analysis [50] which concluded that *Streptococcus* may play a central role in the stability of the lung microbiome. *(iii)* At the cross-kingdom communication level, lung interactions between *Candida* (especially *C. albicans*) and *Pseudomonas* or *Streptococcus* have been described [33,51,52]. There is evidence that *Candida* and *Pseudomonas* coexist in biofilms with both negative and positive association. Roux et al. [51] showed that rats inoculated with *P. aeruginosa* developed pneumonia only in the presence of viable *C. albicans*. *P. aeruginosa* secretes phenazines which are toxic to yeast and hyphal forms of *C. albicans.* It also produces a quorum-

sensing which is similar to farnesol, a molecule secreted by *C. albicans* to regulate hyphal growth (for review: [53]). All these interactions may contribute to the formation of a specific microbiome in CF patients' lungs able to face host-immune response and antimicrobial treatment. Our findings add support to the complex interaction especially during exacerbation between typical pathogens and microbiota, such as the association between *P. aeruginosa* and anaerobes, and/or fungi.

Conclusion

The rationale of this study was whether PMA-pretreatment had an effect on the measured bacterial and fungal compositions, presumably resulting in a more accurate account of the viable microorganisms in CF lung microbiota. While culture- independent methods such as HTS are not able to discriminate dead from viable cells, culture dependent methods allow us to identify viable microorganisms, but not non-cultivable ones. PMA combined with HTS is therefore believed to be a worthy perspective solution that allowed us to show that during acute exacerbation the CF airway microbiome is diverse and largely made up of viable community members. According to our results and published ones [6], PMA-treatment highlights changes in the relative abundance of OTUs in the CF sputum samples which can help for respiratory microbiota analysis without overestimating the abundance of each viable microorganism. Due to its time consumption and cost, this technique should be limited to cases that are difficult to manage. Improvement of the clinical status is the main objective when CF patients chronically colonized with *P. aeruginosa* receive antibiotic treatment to face acute exacerbation. Therefore, including PMA-pretreatment in the HTS sample analysis may be considered as a second line diagnostic tool used when the patient does not respond well to antimicrobial treatment, and/or when biofilm consortium is suspected; these two clinical situations could benefit from an accurate estimation of the "core" or abundant viable community members during exacerbation.

Further studies combining HTS analysis to viable microorganism differentiations are warranted to open new strategies for CF patient management. Given our results and the ability of filamentous fungi to cause acute pulmonary exacerbation, lung mycobiota analysis have to be included in these studies to give a realistic composition of the CF lung microbial community that may shape a local response and explain a specific clinical evolution.

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VI. DISCUSSION

Après avoir établi un travail de synthèse sur le risque fongique dans la mucoviscidose et sur les nouveaux concepts de microbiote et mycobiote pulmonaires, le rationnel de notre étude était de déterminer si un prétraitement des échantillons par PMA modifiait les microbiotes pro- et eucaryote pulmonaires analysés par NGS, en détaillant de façon précise les microorganismes viables dans le microbiote pulmonaire reflétant l'état clinique des patients atteints de mucoviscidose.

Depuis une dizaine année, l'utilisation du PMA pour différencier les cellules vivantes des mortes est régulièrement faite en diagnostic microbiologique (Rogers *et al.*, 2008), hygiène (Vaishampayan *et al.*, 2013; Yáñez *et al.*, 2011; Soejima *et al.*, 2012), ou recherche environnementale (Nocker *et al.*, 2010; Vesper *et al.*, 2008). Le prétraitement par PMA a été appliqué avec succès non seulement pour quantifier des bactéries Gram positives et Gram négatives (Pan and Breidt, 2007; Nocker *et al.*, 2009; Bae and Wuertz, 2009; Nkuipou-Kenfack *et al.*, 2013), mais aussi des micromycètes (Vesper *et al.*, 2008; Shi *et al.*, 2012; Agustí *et al.*, 2013; Andorrà *et al.*, 2010), des virus (Fittipaldi *et al.*, 2010; Sánchez *et al.*, 2012), et des protozoaires (Fittipaldi *et al.*, 2011; Brescia *et al.*, 2009). Alors que le PMA a été combiné avec différentes techniques moléculaires (Pan and Breidt, 2007; Nocker *et al.*, 2007; Rogers *et al.*, 2008; Nocker *et al.*, 2009), peu d'études récentes l'ont été mené avec le séquençage à haut-débit ; la plupart de ces études n'analysaient que la communauté bactérienne dans les échantillons environnementaux (Nocker *et al.*, 2010; Vaishampayan *et al.*, 2013; Chiao *et al.*, 2014), peu d'études ont analysé la communauté fongique ou virale (Venkateswaran *et al.*, 2014; Checinska *et al.*, 2015; Bellehumeur *et al.*, 2015) et des échantillons humains (Rogers *et al.*, 2013; Exterkate *et al.*, 2014). Notre travail visait donc à analyser les mycobiote et bactériote pulmonaires chez les patients atteints de mucoviscidose colonisés par *P. aeruginosa* en utilisant l'approche «PMA - NGS». Elle nous a permis de déterminer l'effet du prétraitement par PMA sur la communauté fongique et la communaté bactérienne co-présente dans les voies respiratoires.

1. Impact de PMA sur l'abondance des communautés bactériennes et fongiques

Dans la mucoviscidose, *P. aeruginosa* est le pathogène le plus fréquent qui provoque des infections chroniques avec des exacerbations aiguës (Høiby *et al.*, 2010; Stenbit and Flume, 2011). Une fois établies, les infections chroniques à *P. aeruginosa* sont difficiles à éliminer en raison notamment de la formation de biofilm (Høiby *et al.*, 2010). Ce germe trouve dans les poumons des

patients une niche composée de bactéries insérées dans une matrice constituée de polysaccharide (notamment d'alginate produite par des souches mucoïdes), de protéines et d'ADN extracellulaire qui lui permet de se développer sous forme de biofilm. Dans ce biofilm, *P. aeruginosa* semble pouvoir interagir avec d'autres espèces bactériennes ou fongiques (Trejo-Hernández *et al.*, 2014; Manavathu *et al.*, 2014), éventuellement constituer un biofilm mixte capable de modifier la progression d'une exacerbation aigue selon le modèle Climax/Attack récemment proposé (Whiteson *et al.*, 2014b; Quinn *et al.*, 2014).

Dans notre étude, l'abondance de *P. aeruginosa* dans l'échantillon traité par PMA était significativement plus faible que dans celui non traité, ce qui correspond aux données publiées sur l'impact de PMA (Rogers *et al.*, 2013; Deschaght *et al.*, 2013). Ce résultat est également en accord avec la capacité de *P. aeruginosa* à former des biofilms dans les poumons des patients qui pourrait contenir une grande proportion de cellules mortes et d'ADN libre (Deschaght *et al.*, 2010, 2013; Tavernier and Coenye, 2015).

Nos résultats de séquençage à haut-débit ont montré que *P. aeruginosa* était le genre bactérien dominant en accord avec notre critère de sélection de patients chroniquement colonisés par *P. aeruginosa*. Ils ont également confirmé les principaux genres bactériens décrits dans la littérature du bactériote respiratoire des patients atteints de mucoviscidose (Madan *et al.*, 2012; Zemanick *et al.*, 2011; Zhao *et al.*, 2012b; Fodor *et al.*, 2012; Goddard *et al.*, 2012; Carmody *et al.*, 2013; Twomey *et al.*, 2013; Lim *et al.*, 2014; Keravec *et al.*, 2015) (Figure 4A - Article 4). Seuls 3 genres fongiques représentaient 94,7% du nombre total des OTUs fongiques. Parmi eux, *Candida* (y compris *C. albicans* (52,3%) est le genre dominant du mycobiote respiratoire dans notre étude (Figure 5A - Article 4). *C. albicans* a été fréquemment isolés dans les expectorations des patients atteints de mucoviscidose. Il existe également des données en faveur d'une co-colonisation de *C. albicans* et *P. aeruginosa* dans les voies respiratoires de patients atteints de mucoviscidose (Leclair and Hogan, 2010). Cependant, son rôle dans la pathogenèse et l'évolution clinique de la mucoviscidose reste à étayer (Touati *et al.*, 2014). Globalement, nos résultats ont confirmé la composition du mycobiote pulmonaire décrit précédemment (Delhaes *et al.*, 2012; Charlson *et al.*, 2012b; van Woerden *et al.*, 2013; Willger *et al.*, 2014). De plus, notre étude a identifié une abondance relative de *Nakaseomyces delphensis* assez importante (11,23%) répartie dans 7 échantillons appartenant à 3 des 5 patients. Cette levure qui a été isolée initialement des figues sèches en Afrique du Sud (Walt and Tscheuschner, 1956), est une levure aérobique typique, non pathogène chez l'homme et proche phylogénétiquement que *Candida glabrata* (Kurtzman, 2003; Correia *et al.*, 2006). À notre connaissance, ce champignon n'a jamais été détecté dans les voies respiratoires humaines. Ce résultat souligne la possible exposition des poumons à des

microorganismes environnementaux et l'importance d'analyser l'ensemble du microbiote pulmonaire incluant notamment le mycobiote.

Nous avons observé que le nombre moyen d'OTUs des échantillons traités avec le PMA (microorganismes viables) était légèrement plus élevés que celui des échantillons non traitées (ADN total) et la taille moyenne des OTUs des échantillons traitées au PMA était légèrement plus courte que celle des échantillons non traités. Plusieurs facteurs ont été récemment proposées comme affectant les résultats d'un prétraitement par PMA tels que le type d'échantillons, la concentration du PMA, le temps d'incubation avec PMA, la source de lumière et le temps d'exposition, la longueur du gène ciblé, le pH du mélange de PCR, … (Nocker *et al.*, 2010; Fittipaldi *et al.*, 2012; Taylor *et al.*, 2014). Le principe du traitement par PMA est basé sur l'intégrité membranaire qui est reconnue comme un déterminant majeur de la viabilité cellulaire. Donc, en couplant le traitement PMA avec d'autres facteurs déterminant de viablité (y compris les activités enzymatique et métabolique) ou avec le traitement de l'échantillon avec une enzyme DNase / protéinase K pourrait aider à une réduction des faux signaux positifs (Chiao *et al.*, 2014). Malgré quelques différences mineures, notre protocole « PMA - NGS » est similaire à ceux publiés (Tableau 1 – page 100). En outre, les estimations du nombre de cellules viables dans nos échantillons étaient de plus de 10^7 cellules/ml, en accord avec le seuil de 10^5 cellules/ml récemment publié pour obtenir l'effet optimal du prétraitement par PMA (Tavernier and Coenye, 2015). La taille moyenne (> 300 pb) des reads des échantillons était suffisamment longue pour présenter une détection précise des cellules viables (Nocker *et al.*, 2010; Martin *et al.*, 2013).

2. Impact de PMA sur la biodiversité des communautés bactériennes et fongiques

S'il n'y a pas de différence entre deux groupes avec ou sans PMA au niveau de la population totale, nous avons observé des différences significatives au niveau des populations bactériennes intermédiaires (Tableau 1 – Article 4). En effet, la biosphère "rare" ou "intermédiaire" est une notion importante en écologie, appliquée souvent aux échantillons d'eau de mer (Sogin *et al.*, 2006; Galand *et al.*, 2009; Pedrós-Alió, 2012; Mangot *et al.*, 2013; Logares *et al.*, 2014; Quero and Luna, 2014). Malgré une définition des « taxons rares ou intermédiaires » variable d'un auteur à l'autre (moins de 1%, moins de 0,1%, ou moins de 0,01%), ces études ont montré que les populations minoritaires variaient plus que les populations majoritaires et qu'elles joueraient un rôle important dans la stabilité de l'écosystème étudié. Ici, notre résultat est conforme à celui de Rogers *et al.*, 2013 puisqu'ils n'avaient pas identifié de différence significative dans la

communauté bactérienne entière mais une influence du prétraitement par PMA sur la composition des taxons «satellites» analysés par NGS.

D'autres études utilisant la combinaison « PMA-NGS » pour comparer les profils bactériens d'échantillons environnementaux ont également montré des changements basés sur l'abondance et/ou les indices de diversité (Nocker *et al.*, 2010; Vaishampayan *et al.*, 2013; Venkateswaran *et al.*, 2014; Chiao *et al.*, 2014). Alors que Vaishampayan et al. [75] ont montré une différence dans la structure des bactériotes d'échantillons environnementaux par PCA, nous avons trouvé à l'inverse un positionnement relativement proche par PCA des couples d'échantillons (Figure 4B et 5B – Article 4). Ces résultats sont comparables aux résultats obtenus sur des échantillons de la cavité buccale (Exterkate *et al.*, 2014).

En ce qui concerne le mycobiote, nous n'avons pas trouvé une différence significative entre les échantillons traités et non traités par PMA (Tableau 1, Figure 5B – Article 4). À notre connaissance, ce travail est la première étude portant sur l'effet du PMA sur le mycobiote respiratoire analysé par NGS, ce qui limite la comparaison et discussion de nos résultats. Cependant, la méthode « PMA-NGS » a été appliquée pour évaluer le mycobiote des échantillons de poussière (Venkateswaran *et al.*, 2014). Les auteurs ont trouvé des différences remarquables entre les échantillons traitées et non traitées par PMA, et ont indiqué la présence d'une grande proportion des genres pathogènes tels que *Aspergillus*, y compris *A. fumigatus* (Venkateswaran *et al.*, 2014). Dans notre étude, *Candida, Malassezia, Alternaria,* et *Fusarium* ont été identifiés mais pas *Aspergillus*. Ceci pourrait être du à la méthode d'extraction de l'ADN (insuffisante pour casser les parois des cellules fongiques) ou aux amorces (incapables d'amplifier le locus ITS2 du genre aspergillaire). Notre méthode d'extraction est vraisemblablement adéquate pour extraire un ADN aspergillaire puisque nous pouvons amplifier l'ADN d'autres genres filamenteux taxonomiquement proche d'*Aspergillus* tel que *Fusarium*. Les amorces utilisées dans cette étude ont été sélectionnées sur leur efficacité à amplifier les loci ITS de diverses espèces fongiques, incluant aussi bien des *Ascomycota*, des *Basidiomycota* et des *non-Dikarya* (Liu *et al.*, 2012; Toju *et al.*, 2012). Ceci suggère qu'*A. fumigatus* n'était pas présent dans nos échantillons comme le confirme la q-PCR (Fréalle *et al.*, 2009). La colonisation fongique des patients de cette étude n'ayant pas été recherchée, nous avons confirmé l'absence de détection d'*Aspergillus* par q-PCR. Enfin, cette absence d'*Aspergillus*, en particulier d'*A. fumigatus* pourrait être expliquée par le fait que *P. aeruginosa* produit des phénazines capables d'inhiber la croissance d'*A. fumigatus* (Briard *et al.*, 2015).

Dans leur ensemble et sous un angle écologique, nous avons rapporté que le nombre des séquences (ou OTUs) de *Pseudomonas* augmentait de 27,8% (sans PMA) à 41,4% (avec PMA),

ce qui est similaire aux résultats de Rogers *et al.*, 2013. En parallèle, le nombre d'OTUs de *Streptococcus*, *Azomonas* et *Candida* a diminué de 29,3% à 25,5%, de 21,6% à 6,6%, et de 71,5% à 69,7%. Comme les patients dans notre étude étaient colonisés chroniques par *P. aeruginosa*, nous suggérons donc que nos résultats expliquent des interactions entre communautés microbiennes dans les poumons:

(i) Les diminution du pH, une concentration faible d'oxygène, la présence d'un mucus épais et la présence des métabolites tels que l'alanine et le lactate favorisent la croissance de *P. aeruginosa* sous forme de biofilm (pour revue voir (Marsland and Gollwitzer, 2014)) qu'un traitement par PMA met mieux en évidence en différenciant les cellules vivantes des mortes.

(ii) Dans de telles conditions optimales pour le développement du *Pseudomonas*, d'autres genres tels que *Streptococcus* et *Azomonas* ont moins de chances de survie conformément à l'hypothèse proposée par (Filkins *et al.*, 2012) où *Streptococcus* joue un rôle dans la stabilité du microbiote du poumon.

(iii) Enfin des communications interspécifiques, entre bactéries et champignons, telles que des interactions entre *Candida* et *Pseudomonas* ou *Streptococcus* ont été décrites (Roux *et al.*, 2009; Méar *et al.*, 2013; Sztajer *et al.*, 2014; Xu *et al.*, 2014). Il a été montré que *Candida* et *Pseudomonas* peuvent coexister dans un biofilm mixte qui favorise ou inhibe le développement de l'un ou l'autre. *P. aeruginosa* sécrète des phénazines qui sont toxiques pour les hyphes de *C. albicans* (Gibson *et al.*, 2009). *P. aeruginosa* produit également un «quorum-sensing» qui est similaire au farnésol, une molécule sécrétée par *C. albicans* pour réguler sa croissance (Peleg *et al.*, 2010). De plus, des souris inoculées avec *P. aeruginosa* n'ont développé une pneumonie qu'en présence de *C. albicans* sous formes de levures vivantes (Roux *et al.*, 2009). Toutes ces interactions pourraient contribuer à la formation d'un microbiote spécifique adapté à la réponse immunitaire de l'hôte et au traitement antimicrobien des patients atteints de mucoviscidose. Ainsi, l'approche « PMA-NGS » reflète probablement plus précisément les interactions physiologiques et métaboliques entre les microorganismes, et donc pourrait expliquer plus clairement des évolutions cliniques différentes de patients atteints de la mucoviscidose répondant plus ou mon bien à la prise en charge thérapeutique (Figure 6 - Article 4).

Cependant, il reste quelques limitations dans notre étude. Premièrement, dans le concept d'étude, le nombre relativement restreint d'échantillons (15 échantillons) nous limitait dans l'analyse statistique (utilisation des tests de Wilcoxon et ANOSIM). Deuxièmement, si l'équipe de Rogers (Rogers *et al.*, 2013) a proposé une méthode de division en groupe "core taxa" et "satellite taxa" utilisant la distribution d'abondance des espèces (ou "species abundance distribution-SAD") (Magurran and Henderson, 2003; van der Gast *et al.*, 2011) dans un travail similaire (basé aussi

sur le PMA) dans la mucoviscidose, nous avons opté pour une répartition de la communauté bactérienne et fongique au niveau des genres en deux parties, selon que l'abondance relative des OTUs est supérieure ou égale à 1% (abondante) et inférieure à 1% (intermédiaire) en accord avec la classification déjà décrite (Goddard *et al.*, 2012). Or, il est difficile de concilier ces taxons aux notions de « core taxa » du microbiote pulmonaire proposé par Rogers *et al.*, 2013. Cette classification correspond à la définition la plus large, regroupant les définitions de "taxon rare" variables d'un auteur à l'autre (Sogin *et al.*, 2006; Pedrós-Alió, 2006; Galand *et al.*, 2009; Youssef *et al.*, 2010; van der Gast *et al.*, 2011; Mangot *et al.*, 2013; Logares *et al.*, 2014; Quero and Luna, 2014).

Malgré ces limitations, un des points remarquables de notre travail est que nous avons étudié pour la première fois l'impact d'un prétraitement au PMA sur le mycobiote pulmonaire humain qui représente un domaine émergent. Avec les articles déjà publiés (Delhaes *et al.*, 2012; Charlson *et al.*, 2012b; van Woerden *et al.*, 2013; Willger *et al.*, 2014), cette étude contribue à améliorer notre compréhension de la composition et de la dynamique du mycobiote co-présent avec le bactériote pulmonaire.

Nous proposons que l'approche « PMA-NGS » qui reflète plus précisément les interactions physiologiques et métaboliques entre les microorganismes vivants, soit réservée à l'étude des mycobiote et bacteriote pulmonaires de patients atteints de mucoviscidose ne répondant plus ou répondant moins bien aux traitements antibiotiques de première intention. En effet, un tel protocole étant couteux et chronophage, nous proposons son intérêt potentiel dans une approche multidisciplinaire du suivi des patients atteints de mucoviscidose, en particulier lors de réponses inadaptées au traitement antimicrobien et/ou lors de suspicion de formation de biofilm (Figure 6 - Article 4). D'autres études basées sur l'utilisation de prétraitement tel que le PMA seront nécessaires pour bien documenter nos connaissances en "omic" et les incrémenter dans la prise en charge des patients.

Tableau 1: Résumé des protocoles publiés de « PMA-NGS »

Premier auteur, année	Type d'échantillon	Concentration finale de PMA (µM)	Temps d'incubation (minutes)	Temps d'exposition à la lumière (minutes)	Taille des gènes ciblés (pb)	Amplicons ciblés	Technologie de NGS
Nocker *et al.*, 2010	Echantillons d'eau marine	50	5	3	210-270	V5-V6 d'ADNr16S	FLX Titanium system
Vaishampayan *et al.*, 2013	Echantillons environnementaux	50	60	3	~ 500	V1-V3 d'ADNr16S	
Rogers *et al.*, 2013	Expectorations des patients atteints de mucoviscidose	50	30	Inconnu	~ 466	V2-V3 d'ADNr16S	
Chiao *et al.*, 2014	Echantillons de filtres d'eau potable	50	15	10	~ 346	V4-V5 d'ADNr16S	
Exterkate *et al.*, 2014	Echantillons buccales humains	50	5	2	Inconnu	V5-V7 d'ADNr16S	
Venkateswaran *et al.*, 2014	Particules de poussière	25	5	15	~ 600	V1-V3 et V3-V5 d'ADNr16S ; locus ITS1	
Checinska *et al.*, 2015	Particules de poussière	25	5	15	~ 600	V1-V3 et V3-V5 d'ADNr16S ; locus ITS1	
Bellehumeur *et al.*, 2015	Echantillons du tissu pulmonaire et de sérum des animaux	100	5	5	2 x 300	PRRSV (virus)	Illumina MiSeq
Notre étude, 2016	Expectorations des patients atteints de mucoviscidose	50	30	10	~ 325	V3-V5 d'ADNr16S ; locus ITS2	Ion Torrent

VII.CONCLUSIONS ET PERSPECTIVES

Pour conclure, le séquençage à haut-débit (NGS) est un outil très prometteur pour étudier la flore poly-microbienne pulmonaire. D'abord essentiellement descriptif, il devrait permettre d'identifier de potentielles interactions intra- et/ou inter-spécifiques (voir inter-règnes) indispensables au maintien du microbiome/microbiote pulmonaire. Cette nouvelle technique devrait nous permettre de mieux comprendre les phénomènes d'infections/colonisation pulmonaires chroniques ainsi que le rôle spécifique des microorganismes fréquemment trouvés dans les poumons des patients. Cette approche doit donc désormais participer à une compréhension plus approfondie des différents phénomènes physiopathologiques impliqués dans les pathologies respiratoires chroniques telle que la mucoviscidose. Cependant, le séquençage à haut-débit est une technique puissante mais pour laquelle des biais peuvent être introduits à de nombreuses étapes méthodologiques. Il nous semble donc important voire nécessaire de vérifier la qualité des résultats à chaque étape et de standardiser au maximum la méthode afin d'être reproductible entre différents centres ou équipes et différentes études.

Durant ces 3 années, nous avons centré nos travaux autour du concept de risque fongique dans la mucoviscidose, notamment autour des concepts de microbiote et mycobiote pulmonaires ainsi que d'une approche expérimentale incluant l'optimisation des étapes « pré-métagénomiques » et l'analyse bioinformatique des données.

Ainsi, dans la démarche expérimentale des études de séquençage haut-débit, l'extraction de l'ADN est aussi un procédé essentiel dont la quantité et la qualité sont crutiales. Certaines bactéries comme par exemple les staphylocoques présentent une paroi (de bactéries Gram positif) plus difficile à lyser que celles des bactéries de Gram négatif (Mahalanabis *et al.*, 2009; Zhao *et al.*, 2012a). De la même façon, l'extraction d'ADN fongique n'est pas aisée, car les parois des champignons et de leurs spores sont également épaisses composées de constituants amorphes résistants aux produits chimiques, enzymatiques et aux perturbations physiques légères (van Burik *et al.*, 1998; Chen *et al.*, 2002; Karakousis *et al.*, 2006). Dans le contexte du microbiote pro- et eucaryotique pulmonaire de la mucoviscidose, cette communauté microbienne complexe va nécessiter une extraction nucléotidique adaptée, optimisée que nous devons étudier (comparaison de différentes méthodes d'extractions d'ADN pour déterminer et proposer celle qui permettra de rendre compte de la façon la plus exhaustive et précise possible de la composition du mycobiote et du bactériote dans les expectorations de patients).

De plus, il existe également plusieurs protocoles pour l'étape de préparation de la librairie métagénomique ce qui entraine une difficulté à comparer les résultats inter-centres. Pour standardiser cette étape, la société Genoscreen a développé leur propre kit d'analyse de la flore bactérienne nommé METABIOTE®. Nous nous sommes orientés vers cette technologie et avons participé en collaboration avec la société Genoscreen au beta-test de leur kit ciblant l'ADNr 16S bactérien (voir l'annexe 2). De la même façon, un kit ciblant l'ADN fongique est en développement.

In fine, la validation de toutes ces étapes devrait permettre à terme de proposer un protocole relativement standardisé d'étude du mycobiote et microbiote respiratoire humain par le séquençage à haut-débit comportant un prétraitement des expectorations avec PMA, une méthode compatible d'extraction d'ADN et les PCRs ciblant les gènes ITS2 et 16S avec les kits METABIOTE®. A moyen terme, ces résultats vont nous permettre d'être plus efficients dans des études prospectives, de grande échelle, multicentriques comme le projet « MucoBacMyco » mené en collaboration entre les Pr Botterel-Chartier et Delhaes. L'objectif du projet « MucoBacMyco » est d'étudier la relevance clinique de l'approche de métagénomique ciblée dans le suivi clinique et le pronostic évolutif des patients atteints de mucoviscidose, en analysant la corrélation du microbiote et mycobiote aux profils clinico-biologique des patients, notamment dans les cas de discordances clinico-biologiques lors des traitements anti-infectieux. En intégrant la dimension écologique, cette approche devrait nous permettre d'appréhender différemment une altération de la fonction pulmonaire ainsi que l'impact du traitement anti-infectieux sur l'écosystème poly-microbien respiratoire dans la mucoviscidose.

L'émergence récente du concept de mycobiote et microbiote pulmonaire ainsi que les connaissances qui en découlent devraient, dans un avenir proche, nous permettre de mieux connaitre la physiopathologie du poumon inflammé/colonisé des maladies respiratoires chroniques telles que la mucoviscidose, et donc de proposer de nouvelles approches thérapeutiques pour cette maladie.

VIII. REFERENCES BIBLIOGRAPHIQUES

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IX. ANNEXES

Annexe 1 : Valorisation du travail

Publications :

1. K Touati, LDN Nguyen, L Delhaes (2014). The airway colonization by opportunistic filamentous fungi in patients with cystic fibrosis: Recent Updates. Curr Fungal Infect, 8(4): 302–311. doi:10.1007/s12281-014-0197-7.

2. LDN Nguyen, E Viscogliosi and L Delhaes (2015). The lung mycobiome: an emerging field of the human respiratory microbiome. Front. Microbiol. 6:89. doi: 10.3389/fmicb.2015.00089.

3. LDN Nguyen, L. Delhaes (2015). Un nouveau concept : Le mycobiome pulmonaire. Médecine Science. Vol 31, n° 11, p. 945-947. doi: 10.1051/medsci/20153111002.

4. LDN Nguyen, P. Deschaght, S. Merlin, A. Loywick, C. Audebert, S. Van Daele, E. Viscogliosi, M. Vaneechoutte, L. Delhaes. Effects of propidium monoazide pretreatment on cystic fibrosis airway mycobiome and bacteriome characteristics. Article original accepté dans PloS ONE avec les révisions mineures.

Travail présenté en communication orale en congrès scientifiques :

1. LDN Nguyen, P. Deschaght, S. Merlin, A. Loywick, C. Audebert, E. Viscogliosi, M. Vaneechoutte, L. Delhaes. Prétraitement par le Propidium monoazide d'expectorations de patients atteints de mucoviscidose modifie les mycobiome et bacteriome de la communauté intermédiaire. Présentation orale accepté au congrès 2ème journée Franco-Maghrébines de Parasitologie et Mycologie, Tunisie, 28-31 Octobre 2015.

Travaux présentés en communication affichée en congrès scientifiques :

1. L Delhaes & LDN Nguyen. Mould and Cystic Fibrosis: What can we learn from studying lung mycobiota? E-poster N°1498. ECCMID congress, Congrès international, Barcelona, 10-13 Mai 2014.

2. LDN Nguyen, R Dassonneville, M Chabé, N Gantois, A Prévotat, T Perez, B Wallaert, C Audebert, A Goffard, E Viscogliosi, L Delhaes. Lung mycobiota from patients with CF: Recent updates and links with other microbial communities. Poster au congrès de la SFMM, Reims, 21-23 Mai 2014.

3. L Delhaes, LDN Nguyen, E Sitterle, E Viscogliosi, F Botterel. Lung mycobiota from patients with cystic fibrosis: Recent updates and links with other microbial communities. Présentation Orale au congrès du WG “Fungal risk in CF", Angers, 5-6 Juin 2014.

4. N Adele-Dit-Renseville, LDN Nguyen, L-E Vandenborght, S Terrat, N Gantois, L Ranjard, L Delhaes, S Ferreira. Metabiote®: Development and application of an integrated solution for microbiota analysis. Poster au congrès du “5th International human microbiome congress” (IHMC), Luxembourg, 31 Mars – 4 Avril 2015.

5. LDN Nguyen, P. Deschaght, S. Merlin, A. Loywick, C. Audebert, E. Viscogliosi, M. Vaneechoutte, L. Delhaes. Prétraitement par le Propidium monoazide d’expectorations de patients atteints de mucoviscidose modifie les mycobiome et bacteriome de la communauté intermédiaire. Poster au congrès de la SFMM, Bordeaux, 20-22 Mai 2015.

6. LDN Nguyen, P. Deschaght, S. Merlin, A. Loywick, C. Audebert, S. Van Daele, E. Viscogliosi, M. Vaneechoutte, L. Delhaes. Propidium monoazide (PMA)-pretreatment of sputum samples impacts the intermediate community of cystic fibrosis (CF) airway mycobiome and bacteriome. Poster au congrès international 38th European Cystic fibrosis society, Bruxelles, 10-13 Juin 2015.

Annexe 2 : Poster présenté au congrès des SFP-SFMM, Reims, France, Mai 2014

Lung mycobiota from patients with cystic fibrosis: Recent updates and links with other microbial communities?

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Abstract :

Recent studies using culture-independent microbial detection methods (Deep-sequencing) enable exploration of microbial community composition in both healthy and abnormal lungs. These methods reveal that cystic fibrosis (CF) airway bacterial communities are more diverse than previously appreciated and vary in both short- and long-term. Each community has its own composition and evolution, unique and specific to each patient. The microbiota dynamics might account for CF disease outcome especially during acute exacerbations. Furthermore, although fungi and viruses are increasingly recognized as important agents in pulmonary exacerbations, only a limited number of small deep-sequencing studies have focused on viruses and phages, and/or fungi. Given the polymicrobial nature of pulmonary infections in CF patients and the recent evidence that fungi may be of clinical relevance in the decline of CF lung function, we developed a high-throughput sequencing approach to extensively explore the diversity and dynamics of fungal and prokaryotic populations in CF upper airways. In particular, we explore links between pulmonary acute exacerbation and moulds (such as *Aspergillus fumigatus* and *Scedosporium*), taking into account the context of CF and the polymicrobial nature of airway community. Methodology and Principal Findings: Pyrosequencing (454 FLX) approach was used to address fungal diversity in lung (i.e. lung mycobiota), as previously described. Sputum samples from CF patients with (11 patients) and without (12 patients) pulmonary exacerbation were compared. Moulds, bacteria and respiratory viruses were identified using conventional methods, RT-PCR and deep-

sequencing approach. Mycobiota plus bacterial microbiome seem to be correlated to lung function and spirometry values (FEV1). *Candida* and *A. fumigatus* were the major taxa isolated in these studies. Results were analysed taking into account biological characteristics of species (or at least genus) that are able to explain interactions between microorganisms. They were discussed considering exacerbation and clinical features. Our approach (based on Principal component analysis (PCA)) confirmed the important role of *Streptococcus* species in increasing lung microbiota diversity, promoting patient stability, and allowed us to conceptualize such interactions of the CF lung environment. To conclude, mycobiota seems to be a dynamic event, part of the overall microbiome, i.e. including bacterial microbiota and virome, plus some potential interaction such as resistosome. We thus interpreted our results to highlight the potential interactions between microorganisms and the role of fungi (such as *A. fumigatus*) in the context of improving survival in CF.

Lung mycobiota from patients with cystic fibrosis: Recent updates and links with other microbial communities

Linh Nguyen Do Ngoc[1], Romain Dassonneville[2], Magali Chabé[1], Nausicaa Gantois[1], Anne Prévotat[3], Thierry Perez[3], Benoit Wallaert[3], Christophe Audebert[2], Anne Goffard[4], Eric Viscogliosi[1], Laurence Delhaes[1,5]

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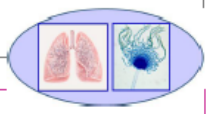


INTRODUCTION & AIM:

✓ Recent studies using culture-independent microbial detection methods (Deep-sequencing) enable exploration of microbial community composition in both healthy and abnormal lungs. These methods reveal that cystic fibrosis (CF) airway bacterial communities are more diverse than previously appreciated and vary in both short- and long-term. **Each community has its own composition and evolution, unique and specific to each patient. The microbiota dynamics might account for CF disease outcome especially during acute exacerbations.** Furthermore, although fungi and viruses are increasingly recognized as important agents in pulmonary exacerbations, only a limited number of small deep-sequencing studies have focused on viruses and phages, and/or fungi.

✓ Given the polymicrobial nature of pulmonary infections in CF patients and the recent evidence that fungi may be of clinical relevance in the decline of CF lung function, **we developed a high-throughput sequencing approach to extensively explore the diversity and dynamics of fungal and prokaryotic populations in CF upper airways.**

➔**Specifically, we explore links between pulmonary acute exacerbation and moulds (such as *Aspergillus fumigatus* and *Scedosporium*), taking into account the context of CF and the polymicrobial nature of airway community.**

- ❖ Pulmonary exacerbation = key event in CF lung alteration
- ❖ What is the role of MBPA (allergic bronchopulmonary mycosis) in such exacerbation?
- ❖ With the idea of deciphering the place of *Aspergillus/Scedosporium*

MATERIAL & METHODS:

• Population and samples:

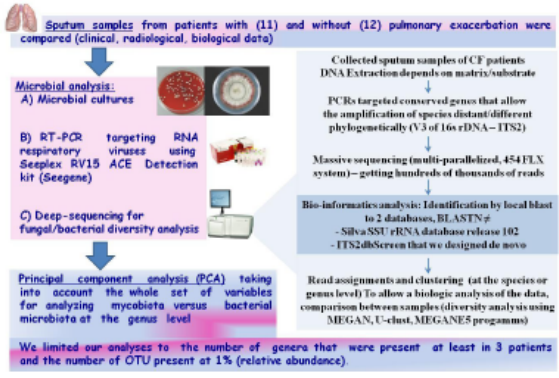
Sputum samples from CF patients with (11 patients) and without (12 patients) pulmonary exacerbation were compared

• Methods:

➔ *Microbiological analysis:* Moulds, bacteria and respiratory viruses were identified using conventional methods, RT-PCR and deep-sequencing approach.

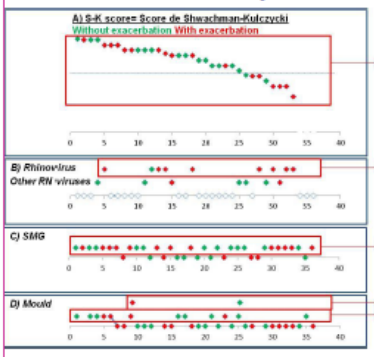
➔ *Pyrosequencing (454 FLX) approach* was used to address fungal diversity in lung (i.e. lung mycobiota), as previously described *[Delhaes et al, 2012]* .

➔ *Data were analysed according to the following workflow*



RESULTS:

• Conventional microbial analysis:



No correlation between SK-score and pulmonary exacerbation

We confirmed the significant association between HRV and pulmonary exacerbation *[Goffard et al. J Clin Virol. 2014 Feb 25]*

No association between Milleri group Streptococcus and pulmonary exacerbation

Only 2 patients colonized with *Scedosporium apiospermum*

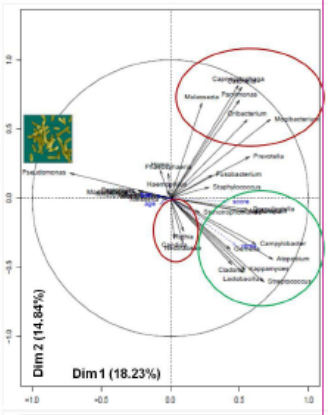
No association between *A. fumigatus* and exacerbation

• Deep-sequencing results:

- ✓ 953 999 reads size from 315 to 468 pb - 2/3 16s rDNA + 1/3 ITS2
- ✓ Optimal rarefaction curves
- ✓ CPA and modelization under process

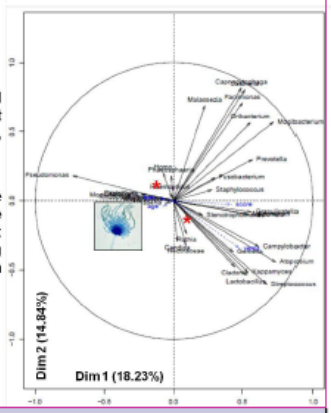
✓ **Pseudomonas**

- is alone in agreement with published data *[Zemanick et al. 2013]*
- not correlated with "Malassezia plus Prevotella group" *[Zemanick et al 2013]*
- neither with the "Candida plus Rothia group" (which is not well explained by our axes since the arrows are short)
- but is negatively correlated with the "group of oral flora including streptococcus plus some environmental fungi", as well as FEV1 – SK-score *[Zemanick et al. 2013]*



✓ **Aspergillus**

- Unfortunately, our PCA model explained poorly this mold (short arrows, anti-correlated to SK-score, FEV1,),
- Neither exacerbation status: There was no differentiation between the group of patients with and without pulmonary exacerbation (according to PCA-barycenter (*) of each patient group)



DISCUSSION & CONCLUSION:

• Using deep-sequencing and PCA approaches, we confirmed the relation between *Pseudomonas aeruginosa* and alteration of respiratory function as well as an anti-correlation between *P. aeruginosa* and bacteria of the mouth community as previously described *[Zemanick et al. 2013]*. Most of current studies have focused on bacterial microbiota in CF patients while viruses and/or moulds have been reported as pathogens *[Alouat et al 2014]*.

• While Rhinoviruses (recombined HRV-Ca) were significantly associated to acute pulmonary exacerbation ($p = 0.027$) *[Goffard et al 2014]*, we didn't observe any correlation between *Aspergillus fumigatus* and exacerbation in our population.
This might reflect the fact that ***Aspergillus* spp. especially *A. fumigatus* isolated from respiratory secretions is often a dilemma for the CF clinician in terms of clinical relevance and treatment** *[Liu et al. 2013]*.

• We currently continue the statistical analysis by focusing on *Streptococcus* species and less abundant (but more diverse) components of the mycobiota (rare biosphere - <0.1%). Larger studies are also required to deeply analyze the role of each microorganisms (bacteria, viruses and fungi), in combination with a more ecological analysis *[Whiteson et al. 2014]*.

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Annexe 3 : Poster présenté au congrès du "5th International human microbiome congress" (IHMC), Luxembourg, Avril 2015

METABIOTE®: Development and application of an integrated solution for microbiota analysis

Nathalie Adele-dit-Renseville[1], **Do Ngoc Linh Nguyen**[2], Louise-Eva Vanderborght [1], Sébastien Terrat [4], Nausicaa Gantois [2], Lionel Ranjard [4], Laurence Delhaes[2,3], Stéphanie Ferreira[1]

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Abstract:

The growing need to survey the tremendous microbial diversity in a culture independent manner has led to the development of molecular methods through sequence profiling of phylogenetically conserved genes such as 16S rDNA, in scientific field like ecology, agronomy, biotechnology, plant, animals and of course Human Health. Next--generation sequencing (NGS) technologies providing unprecedented throughput of data, are now used routinely to assess bacterial community composition in complex samples. Consequently, many scientific or clinical studies have been, and are still performed with these NGS technologies. However, several protocols for amplicon library realization exist and are widely used to perform these analyses whereas no study has looked at their respective impact on taxonomical description, relative abundances of taxa, diversity and richness indexes. To address these issues, and starting from one unique sample of soil that presents the highest microbial complexity, we have performed a comparison of two frequently used NGS--amplicon library preparations (direct PCR and ligation) that have led us to develop our own solution named METABIOTE®

The data presented here will show that in house protocols induce biases between samples and impact on taxonomical definition and the observed relative abundances of taxa while our own

solution Metabiote® greatly improve these data. Moreover, our internal protocol generates better diversity and richness indexes indicating that routine protocols can underestimate the complexity of a bacterial community. Our solution has now been tested on several type of human microbiota (faeces, biopsy, skin, sweat, saliva, and sputum samples…) with success and a concrete application on the human respiratory microbiota in the context of Cystic Fibrosis will be presented.

This solution is accessible through our services platform on both 454-Roche (GS Junior, GS FLX) and Illumina (MiSeq2*300pb) and is now available under ready-to-use kits (Metabiote® kits) with the associated fully automated pipeline (Metabiote Online®) for raw data analysis. This bioinformatics pipeline and its web interface allow the users to be autonomous for the analysis and interpretation of their data without any particular IT investment. The first Metabiote® kits have been designed for amplicon libraries preparation targeting 16S rDNA V3V4 or V4V6 regions that can be sequenced using GS Junior & GS FLX sequencers (Roche Diagnostics). Additional kits targeting fungi communities (mycobiome component) and other sequencing platforms are currently under validation.

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METABIOTE®: DEVELOPMENT AND APPLICATION OF AN INTEGRATED SOLUTION FOR MICROBIOTA ANALYSIS

Nathalie ADELE-DIT-RENSEVILLE[1], Do Ngoc Linh NGUYEN[2], Louise-Eva VANDENBORGHT[1], Sébastien TERRAT[4], Nausicaa GANTOIS[2], Lionel RANJARD[4], Laurence DELHAES[2,3], Stéphanie FERREIRA[1]

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[3] *Department of Parasitology-Mycology, Regional Hospital Center, Faculty of Medicine, Lille, France*
[4] *GenoSol Platform, INRA, UMR 1347 Agroécologie, Dijon, France.*

INTRODUCTION

The growing need to survey the tremendous microbial diversity in a culture independent manner has led to the development of molecular methods through sequence profiling of phylogenetically conserved genes such as 16S rDNA, in scientific field like ecology, agronomy, biotechnology, plant, animals and of course Human Health. Next-generation sequencing (NGS) technologies providing unprecedented throughput of data, are now used routinely to assess bacterial community composition in complex samples. Consequently, many scientific or clinical studies have been, and are still performed with these NGS technologies. However, several protocols for amplicon library realization exist and are widely used to perform these analyses whereas no study has looked at their respective impact on taxonomical description, relative abundances of taxa, diversity and richness indexes. To address these issues, and starting from one unique sample of soil that presents the highest microbial complexity, we have performed a comparison of two frequently used NGS-amplicon library preparations (direct PCR and ligation) that have led us to develop our own solution named METABIOTE®.

METABIOTE® SOLUTION

LIBRARY PREPARATION: In the context of cohorte analyses and samples comparison, it is crucial to minimize biais that could occur during amplicon library preparation. Indeed, starting with DNA extracted from one unique sample of soil (know to have the highest microbial community complexity), we have prepared several identical libraries with only distinct indexes following two classical protocol (Direct PCR and Ligation) and its internal Metabiote® Protocol. Figure 1 clearly demonstrated the impact of indexing step on the observed relative abundance of taxa at the phylum level starting from one unique sample (Figure 1A and 1B). On the contrary, Metabiote® shows a clear homogeneity in its results with no impact of the indexing step (Figure 1C).

ANALYSIS AND OTU ASSIGNATIONS: Metabiote is associated fully automated pipeline (Metabiote Online®) for raw data analysis. This bioinformatics pipeline and its web interface allows the users to be autonomous for the analysis and interpretation of their data with no particular IT investment.

DIVERSITY INDEX: Shannon indexes were calculated for each indexed-library and represented with a box-plot graph in order to compare the three different NGS-amplicon library protocols. Figure 2 shows that Direct PCR and Ligation methods generate similar Shannon indexes. Nevertheless, Metabiote® protocol gives access to a slightly higher bacterial diversity information compared to the two others classical protocol, demonstrating its higher efficiency.

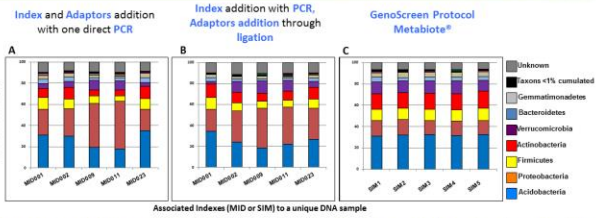
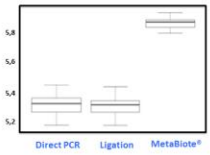


Figure 1: Impact of three different 16S-amplicon library preparation methods on the observed relative abundance of bacterial taxa

Figure 2: Shannon diversity Index obtained with the three different protocols of 16S-amplicon Library preparation



CONCLUSION: Metabiote® is an optimized and standardized process for 16S-amplicon library preparation that induces no bias between samples and give access to a higher diversity than classical protocols. Metabiote® is available on our platform as a service but also as user-friendly kits.

CLINICAL APPLICATION

Introduction

Recurrent pulmonary infections, especially acute exacerbations are the most severe problems leading to the lung function decline and the high mortality in cystic fibrosis (CF).
This study was conducted on 6 sputum samples isolated from 4 CF patients.
All of them were homozygous or heterozygous for the Delta F508 mutation, followed up at Lille and Toulouse hospitals (France) and colonized with *Scedosporium sp.* The patients 1 to 3 were clinically stable at the sampling time. Sputum samples A, B and C were collected from patient 4; A and B were sampled during stable clinical status, while C was sampled during exacerbation.
DNA extraction was performed with MoBio extraction kit according to the manufacturer's instruction with an additional lysis step. DNA samples were processed with Metabiote® V3V4 kit as indicated in the manual and Mock community, provided in the kit as positive control, was processed in duplicate. Libraries were equimolary pooled, amplified by emPCR and sequenced on one eighth PicotiterPlate (PTP) according to Roche Manuals, on a GsFLX system.

Lung microbiota profiles in CF

First, we observed that results on Mock community duplicate showed very similar relative abundance profiles proving the robustness of the kit.

Bacterial profiles were globally different between the four CF patients during stable phase, excepted for patient 3 and 4.

The focus on patient 4 showed that bacterial profiles during stable phase were similar, while bacterial profil **during exacerbation was notably different and more diverse**. The metagenomic data showed that the core members of this bacterial community was mainly anaerobes, which are hypothesized to cause acute exacerbations by producing elements involved in fermentation in a recent publication (Quinn RA, et al).

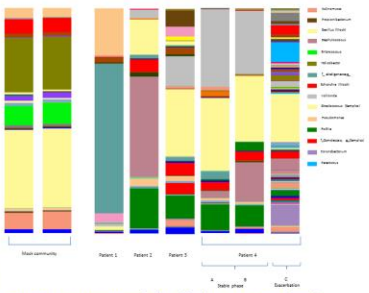


Figure 4: Histogramm of identified OTUs at genus level

Microbial diversity and clinical status

The lung function of patients is mesured principally by spirometry test including forced expiratory volume in 1 second (FEV1) and forced vital capacity (FVC) values. The FEV1 value is one of predicated factors for lung function decline and is altered during acute exacerbation in CF.Patients with FEV1 less than 40% are considered as having a severe lung function decline (Döring G et al, ; Smyth A.R. et al).

Here, we observed the relationship between the spirometric values and the Chao 1 and Shannon indexes, which are commonly used as estimators to evaluate the richness and biodiversity of a microbial community.
We observed a reduction of diversity indexes (their values corresponds to the spot sizes) associated with a lung function decline expressed by decreased spirometric values.

This results agrees with previous publications (Fodor A, et al. ; Delhaes L. et al) which confirm that the profile alteration of CF lung microbiota could be an indicator for poor lung function and poor clinical status of the patients

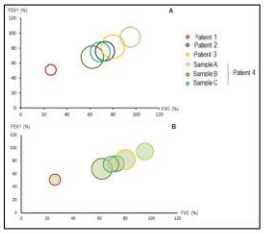


Figure 5: Relationship between the Chao 1 index (A) and Shannon index (B) of lung bacteriome diversity and the lung function (expressed by FEV1 and FVC values in %).

Conclusion

Our findings along with others studies (Fodor A, et al. ; Quinn RA, et al.) from metagenomic analysis are therefore crucial to open new possibilities and strategies for CF patient therapeutic management.
In this context, all these results confirmed that the Metabiote kit is well adapted for analyzing the CF lung bacterial microbiota by deep-sequencing method.

Acknowlegements
These data are part of an ongoing collaborative study. We would like to thank Caroline Thumerelle, Nathalie Wizla, Dominique Tuck, Sophie Cassaing, Judith Fillaux, François Brémont and Marlène Murris for their help to complete this work

Annexe 4 :

Poster présenté au congrès de la SFMM, Bordeaux, 20-22 Mai 2015.

Poster présenté au congrès ECFS 38ème, Bruxelles, Belgium, Juin 2015.

Poster présenté à la "Journée André Verbert", Université Lille 2, Septembre 2015.

Présentation orale au congrès 2ème journée Franco-Maghrébines de Parasitologie et Mycologie, Tunisie, 28-31 Octobre 2015.

Propidium monoazide (PMA) sample pretreatment impacts the abundance of rare populations in high-throughput sequencing analysis of CF lung mycobiome and acteriome

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Abstract :

Propidium monoazide **(**PMA) pretreatment has increasingly been used in molecular biology for detecting viable pathogens. Our study aims to determine whether a PMA pretreatment in high-throughput sequencing (HTS) could provide a more realistic picture of lung mycobiome and bacteriome from cystic fibrosis (CF) patients, and consequently reflect more closely the clinical status of patients.

We compared HTS data of bacteriome and mycobiome of 15 sputum samples from 5 CF patients that were characterized using the Ion Torrent technique with and without prior PMA treatment of the DNA-extracts.

PMA pretreatment had no significant effect on the entire and abundant bacterial community (genera expressed as operational taxonomic unit with a relative abundance of ≥1%) but caused a significant difference in the rare biosphere community (< 1%) when analysing the alpha biodiversity Simpson index (p=0.029). Similarly, regarding beta diversity (non-metric Bray-Curtis dissimilarity analysis), the rare phylotypes also differed more dramatically than the total and abundant ones. Regarding the mycobiome data, there was no difference between PMA-treated and untreated samples.

To conclude, PMA pretreatment seems to change the relative abundance of bacteria, especially in the rare populations, but not fungi. Given such a cumbersome protocol (PMA pretreatment coupled with HTS), we discuss its potential interest within the follow-up of CF patients. As only few studies suggested the use of this protocol in the characterization of the bacteriome may be clinically relevant, further studies using PMA pretreatment are warranted to improve our “omic” knowledge.

Propidium monoazide (PMA)-pretreatment of sputum samples impacts the intermediate community of cystic fibrosis (CF) airway mycobiome and bacteriome

Linh Do Ngoc NGUYEN[1], Pieter DESCHAGHT[2], Sophie MERLIN[3,4], Alexandre LOYWICK[3,4], Christophe AUDEBERT[3,4], Sabine VAN DAELE[5], Eric VISCOGLIOSI[1], Mario VANEECHOUTTE[2], Laurence DELHAES[1,6]

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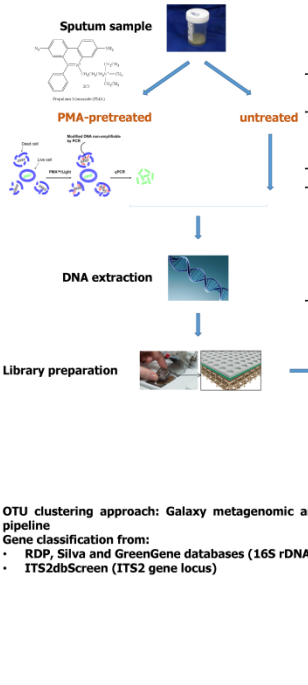
INTRODUCTION & OBJECTIVES

PMA-pretreatment has increasingly been used for selectively detecting viable pathogens in given samples. Recently, few studies showed that PMA-pretreatment could impact the bacterial community profiles obtained by high-throughput sequencing (HTS), which may be clinically relevant *[Rogers et al., 2013]*.

Our study **aims to determine whether the PMA-pretreatment had an effect on the CF airway mycobiome and bacteriome** analyzed by HTS, resulting in a more accurate quantification of the viable fungi and bacteria the CF airway microflora.

MATERIALS & METHODS



- ***Population & sampling:*** 15 sputum samples from 5 CF patients followed-up at Ghent hospital chronically colonized with *P. aeruginosa*.
- ***P. aeruginosa quantification by cultures***
- ***PMA cross-linking*** as previously described *[Deschaght et al., 2013]* before DNA extraction.
- ***qPCR targeting P. aeruginosa***
- ***HTS method:*** Ion Torrent (Life Technologies) system characterizing the bacteriome (targeted 16S rDNA gene variable regions V3–V5) and mycobiome (targeted ITS2 gene locus)
- ***Bioinformatic analysis***

• **Abundant phylotypes (or communities): genera with a relative OTU abundance ≥ 1%**
• **Intermediate phylotypes: genera with a relative OTU abundance <1%.**

RESULTS

Abundance comparison

✓ ***Pseudomonas qPCR & cultures results***

The mean abundance of *P. aeruginosa* cells in PMA-untreated samples using qPCR (total DNA, 7.44 ± 9.21 x 10^7 cells/ml) was significantly higher than that of PMA-treated samples (viable bacteria, 5.17 ± 6.5 x 10^7 cells/ml) and that of cultures (2.91 ± 3.98 x 10^7 cells/ml) (Fig. 1) (paired-sample Wilcoxon test).

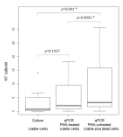


Fig. 1. Comparison of the enumeration of *P. aeruginosa* cells between cultures and qPCRs of PMA-treated and untreated sputum samples

✓ ***HTS results***

- Bacteriome: 14 paired samples - 763,802 reads - 114 genera (Fig. 2A)
- Mycobiome: 10 paired samples - 655,284 reads - 90 genera (Fig. 2B)
- The microbial metacommunity structure between PMA-pretreated and -untreated samples remains virtually stable (Fig. 2).
- No significant difference in the number of reads between the 2 groups.

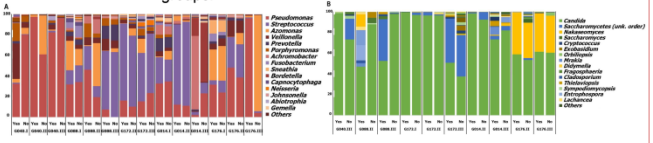


Fig. 2: Relative abundance of bacterial (A) and fungal (B) genera of each sample ("yes" or "no" indicates the samples with or without PMA-pretreatment) (In each case, the first 5 most prevalent genera are listed)

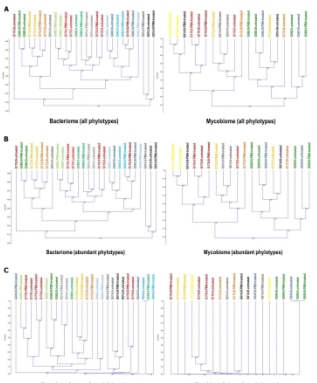


Fig. 3: Dendograms representing the similarity between the composition of bacteriome and mycobiome from individual samples in all (A), abundant (B) and intermediate (C) phylotypes. Clustering is based on Bray Curtis similarity (bootstrap 10,000 replicates).

Biodiversity comparison

✓ ***Alpha diversity***

- Bacteriome: No significant differences between the two groups at the *entire & abundant community levels.*

  Significant difference at the intermediate community based on **Simpson index** (***p=0.029***) and a trend toward significance based on **Shannon index** (***p=0.057***).
- Mycobiome: No significant difference was observed.

✓ ***Beta diversity***

- Both bacteriome and mycobiome: **Beta diversity results confirmed alpha diversity ones** (Fig. 3)

  Only 2 pairs of bacteriome samples and no mycobiome samples still exhibited such similarity at the intermediate community level (Fig. 3C). These results were also confirmed by ANOSIM (Analysis of Similarity) and PCA (Principal Component Analysis).

DISCUSSION & CONCLUSION

- Chronic microbial airway colonization, especially with *P. aeruginosa* is a major problem in CF patients due to antibiotics resistance often caused by biofilm formation. Recently, independent-culture methods such as HTS has become a perspective method for the analysis of CF airway microbiome, but could not discriminate the dead cells from viable ones in samples. In this context, PMA-pretreatment might be a promising approach.
- Our study showed that PMA-pretreatment changed the relative OTUs abundance and biodiversity of CF lung bacteriome and mycobiome, especially in the intermediate communities. However, given such a cumbersome and costly protocol, we discuss its potential interest in the follow-up of CF patients, especially **when the patient do not respond well to antimicrobial treatment, and/or when biofilm consortium is suspected as proposed in the multidisciplinary management of acute exacerbation in CF using HTS (Fig. 4).**
- Most current knowledge on microbial diversity in CF airway concern bacteria, whereas the mycobiome have received little attention *[Nguyen et al., 2014]*. This study provides the first opportunity to focus on PMA-pretreatment effect on the **mycobiome and its co-presence with the bacteriome in human airways.**
- Further HTS studies using PMA-pretreatment are warranted to improve our "microbio-omic" knowledge.

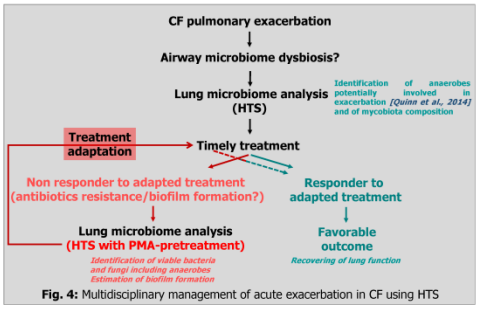


Fig. 4: Multidisciplinary management of acute exacerbation in CF using HTS

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Annexe 5 : Certains indices de diversité les plus couramment utilisés dans la littérature :

L'indice de diversité Shannon est basé sur :

H' = - Σ ((Ni / N) * log2 (Ni / N))

Ni : nombre d'individus d'une espèce donnée, i allant de 1 à S (nombre total d'espèces).

N : nombre total d'individus.

Cet indice peut varier de 0 à 1, H' est minimal (=0) si tous les individus de la population appartiennent à une seule et même espèce, H' est également minimal si, dans une population chaque espèce est représentée par un seul individu, excepté une espèce qui est représentée par tous les autres individus de la population. L'indice est maximal quand tous les individus sont répartis d'une façon égale sur toutes les espèces. Insensible à la richesse spécifique, il est très utile pour comparer les dominances potentielles entre les échantillons.

L'indice de Simpson mesure la probabilité que deux individus sélectionnés au hasard appartiennent à la même espèce, basé sur :

D = Σ Ni(Ni-1)/N(N-1)

Ni : nombre d'individus de l'espèce donnée.

N : nombre total d'individus.

L'indice de diversité de Simpson = 1-D

Cet indice aura une valeur de 0 pour indiquer le maximum de diversité, et une valeur de 1 pour indiquer le minimum de diversité. Dans le but d'obtenir des valeurs «plus intuitives», on peut préférer l'indice de diversité de Simpson représenté par 1-D, le maximum de diversité étant représenté par la valeur 1, et le minimum de diversité par la valeur 0. Il faut noter que cet indice de diversité donne plus de poids aux espèces abondantes qu'aux espèces rares. Le fait d'ajouter des espèces rares à un échantillon, ne modifie pratiquement pas la valeur de l'indice de diversité.

L'indice de diversité Bray Curtis :

La distance de Bray-Curtis est un indice permet d'évaluer la dissimilarité entre deux échantillons donnés, en terme d'abondance d'espèces présentes dans chacun de ces échantillons.

Voici l'équation permettant le calcul de la distance de Bray-Curtis : ici, deux échantillons j et k sont comparés

$$BC_{jk} = 1 - \frac{2\sum_{i=1}^{p} min(N_{ij}, N_{ik})}{\sum_{i=1}^{p}(N_{ij} + N_{ik})}$$

N_{ij} est l'abondance d'une espèce i dans l'échantillon j et N_{ik} est l'abondance de la même espèce i dans l'échantillon k. Le terme min(…,…) correspond au minimum obtenu pour deux comptes sur les mêmes échantillons. Les sommes situés au numérateurs et dénominateur sont réalisées sur l'ensemble des espèces présentes dans les échantillons.

L'indice de dissimilarité de Bray-Curtis est compris entre 0 (les deux échantillons ont la même composition) à 1 (les échantillons sont totalement dissemblables). Sur les sites où BC est intermédiaire (par exemple BC = 0,5) cet indice diffère des autres indices couramment utilisés.