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Non-small cell lung cancer, immunity and microbiota: laying ground for the gut-lung-lung cancer axis in human subjects

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UMR 1019 INRA – Université Clermont Auvergne

THÈSE présentée par :

Rea BINGULA

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Non-small cell lung cancer, immunity and microbiota: laying ground for
the gut–lung–lung cancer axis in human subjects

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SA Greentech

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“People are guests in our story, the same way we are guests in theirs. But we all meet each other for a reason because every person is a personal lesson waiting to be told.” – Lauren Klarfeld

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Thesis context

Born and raised in Zagreb, Croatia, I obtained double Master diploma in Biochemistry, Biotechnology and Molecular Biology from joint collegium of University of Zagreb and University of Orléans in 2015. In January 2016, I began the thesis project in Clermont Ferrand, France, under codirection of Prof Edith Filaire and Prof Marc Filaire in the scope of the fellowship CIFRE with SA Greentech (Saint-Beauzire, France).

Due to its multidisciplinary (microbiology, thoracic surgery, immunology, oncology), the thesis project was ongoing in multiple sites and involved interactions with different teams and specialties. The following non-exhaustive list summarises the main collaborating institutions and the person in charge:

- Centre Jean Perrin: Thoracic Surgery Department (Prof Marc Filaire, MD), Clinical Research (Mrs Emilie Thivat, PhD), Pathology Department (Dr Nina Radosevic-Robin, MD),
- University Clermont Auvergne: UMR1019 INRA/UCA – Laboratory of Biochemistry, Molecular Biology and Nutrition (Prof Marie-Paule Vasson, PhD),
- INRA Centre Auvergne Rhône Alpes – UMR0454 MEDiS (Mrs Annick Bernalier-Donadille, PhD),
- SA Greentech (Mr Jean-Yves Berthon, PhD).

Abstract

Lung cancer is the main cause of death by cancer worldwide. Despite the variety of available treatments, including surgery, chemotherapy, radiotherapy, and immune therapy, the average 5-year survival is 60%. One of the underlying reasons is a very high variability in patients' susceptibility to treatment, explained by genetic background and since recently – our microbiota. The term microbiota includes bacteria, archaea, fungi, viruses and protists that inhabit our organism. The studies in animal models show that the gut microbiota (focused on bacteria) has a crucial role in host's responsiveness to therapy through the stimulation of immune system. In this light, several “communication axes” between the gut and distal tumour sites have started to develop, including the “gut-lung” axis. However, the resident microbiota in the lungs that could directly influence the tumour response and interact with the gut microbiota has been scarcely characterised. To enable further development of the idea of the “gut-lung-lung cancer” axis, we included 18 non-small cell lung cancer (NSCLC) patients eligible for surgery and analysed the microbiota from four different lung samples (non-malignant, peritumoural and tumour tissue and bronchoalveolar lavage fluid; BAL), saliva and faeces by high-throughput sequencing. We also analysed several immune markers, as lymphocytic tumour infiltrate, Th and neutrophil profiles and cytokines in BAL and blood, and inflammatory markers in faeces along with short-chain fatty acids. Focusing first on the lungs, we show that BAL microbiota represents a significantly distinct community compared to lung tissue microbiota by providing detailed characterisation of the four different lung samples. Since tumours in lower lobes are reported as the ones with the worse prognosis, we investigated how the lobe location affected the microbiota composition. Peritumoural tissue and BAL microbiota were identified as the most affected in both abundance and diversity, and tumour as the least affected. However, phylum *Firmicutes*, previously reported as elevated in chronic obstructive pulmonary disease compared to controls, was found more abundant in microbiota from lower lung lobes. Therefore, we propose that both increase in *Firmicutes* and extensive changes in peritumoural tissue could be associated to increased aggressiveness of the lower lobe tumours. Next, we show that the presence of metastatic lymph nodes (LN), negative prognostic marker in NSCLC, significantly influence the local tissue microbiota in relation to its respiratory profile. We reported that anaerobic bacteria were more abundant within the tumour in the presence of metastatic LN, and aerobic bacteria within the one without it.

Abstract

Moreover, exactly inverse was observed for the same bacteria in extratumoural tissues. Along with migratory hypothesis depending on the bacterial preference for growth conditions shaped by tumour's features, we propose several biomarkers for detection of metastatic LN that might facilitate their detection without imposing LN biopsy. Finally, we showed that BAL microbiota is the most associated to the local immune response and independent of the presence of metastatic LN. Future research will focus on the exploration of the interaction between the lung microbiota, systemic immunity and the gut microbiota.

- Key words: non-small cell lung cancer, lung microbiota, immune response, metastatic lymph nodes, tumour lobe

Résumé

Le cancer du poumon est la principale cause de décès par cancer dans le monde. En dépit de la variété de traitements disponibles, tels que la chirurgie, la chimiothérapie, la radiothérapie et l'immunothérapie, la survie moyenne à 5 ans est de 60 %. L'une des raisons sous-jacente est une très grande variabilité de réponse au traitement, expliquée par les antécédents génétiques du patient et depuis peu par son microbiote. Le terme « microbiote » regroupe les bactéries, les archées, les champignons, les virus et les protistes qui colonisent notre organisme. Des études utilisant des modèles animaux montrent que le microbiote intestinal joue un rôle crucial dans la réponse de l'hôte au traitement, via la stimulation du système immunitaire. Dans ce contexte, plusieurs « axes de communication » entre le site intestinal et les sites tumoraux distaux commencent à émerger, y compris l'axe « intestin-poumon ». Cependant, le microbiote pulmonaire, qui pourrait directement influencer la réponse tumorale et interagir avec le microbiote intestinal, est pour l'heure peu caractérisé. Afin de développer cette idée d'un axe « intestin, poumon et cancer du poumon », nous avons inclus dans notre étude 18 patients atteints d'un cancer du poumon non à petites cellules (CBNPC) admissibles à la chirurgie. Nous avons analysé leurs microbiotes par séquençage à haut débit à partir de quatre échantillons différents de poumon (tissu sain, tissus péri-tumoral et tumoral et fluide de lavage broncho-alvéolaire LBA) mais également à partir d'échantillons de salive et de fèces. Nous avons également analysé plusieurs marqueurs immunitaires (infiltration lymphocytaire des tumeurs, profils Th et neutrophiles, cytokines dans le LBA et le sang), des marqueurs inflammatoires et enfin les acides gras à chaînes courtes dans les fèces. Une caractérisation détaillée de ces quatre types d'échantillons de poumons nous a permis de montrer que le microbiote du LBA présente une communauté nettement distincte de celle du tissu pulmonaire. Les tumeurs des lobes inférieurs prédisant le plus mauvais pronostic, nous avons décidé d'étudier le lien entre l'emplacement des tumeurs et la composition du microbiote. Les microbiotes du tissu péri-tumoral et du LBA ont été identifiés comme étant les plus impactés en terme d'abondance et de diversité ; la tumeur est quant à elle moins impactée. Cependant nous avons observé que le phylum des *Firmicutes*, décrit comme étant élevé dans les maladies pulmonaires obstructives chroniques, est plus abondant dans le microbiote des lobes inférieurs du poumon. Par conséquent, nous pouvons émettre l'hypothèse que l'augmentation des *Firmicutes* et les variations importantes du microbiote dans le tissu péri-tumoral pourraient être associés à une agressivité accrue des tumeurs du lobe inférieur. Nous avons ensuite démontré que la présence

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de ganglions lymphatiques (GL) métastatiques, marqueur d'un pronostic négatif dans le NSCLC, influence considérablement le microbiote local de par le profil respiratoire du tissu. Nous avons en effet observé que les bactéries anaérobies étaient plus abondantes dans les tumeurs en présence de LN métastatiques. Les bactéries aérobies sont quant à elles plus représentées dans les tumeurs sans GL métastatiques. Nous avons cependant observé la situation inverse dans les tissus extratumoraux. L'hypothèse avancée est celle d'une migration bactérienne en fonction des préférences de conditions de croissance, directement liées aux caractéristiques de la tumeur. Ceci nous permet de proposer plusieurs biomarqueurs pour la détection de GL métastatique, facilitant ainsi leur détection sans imposer de biopsie. Enfin, nous montrons que le microbiote du LBA est d'avantage associé à la réponse immunitaire locale et est indépendant de la présence de GL métastatique. Les recherches à venir porteront sur l'exploration de l'interaction entre le microbiote pulmonaire, l'immunité systémique et le microbiote intestinal.

- Mots clés : cancer du poumon non à petites cellules, microbiote pulmonaire, réponse immunitaire, ganglions lymphatiques métastatiques, lobe tumoral

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Abbreviations

A

ADK – adenocarcinoma
APC – antigen-presenting cell

B

BAL – bronchoalveolar lavage
Bf – *Bacteroides fragilis*
BPT – background predominant taxa

C

CCR – chemokine receptor
COPD – chronic obstructive pulmonary disease
CRC – colorectal cancer
CTLA-4 – cytotoxic T lymphocyte antigen 4
CTX – cyclophosphamide
CXCL – chemokine (C-X-C motif) ligand

D

DAMPs – damage-associated molecular patterns
DC – dendritic cells

F

FOXP3 – fork-head box protein 3

G

GATA3 – GATA binding protein 3
GF – germ-free
GM-CSF – granulocyte-macrophage colony-stimulating factor
GPR – G-protein coupled receptor

H

HIF-1 – hypoxia-inducing factor 1
HMP – Human Microbiome Project

I

IBD – inflammatory bowel disease
IEL – intraepithelial lymphocytes
IEC – intestinal epithelial cells
IL – interleukin
IFN – interferon

K

KRAS - Kirsten ras oncogene homolog

L

LC – lung cancer
LN – lymph nodes
LPS – lipopolysaccharide
LT – larger tumour
LUNG.DP – non-malignant distant piece/specimen
LUNG.PT – peritumoural tissue
LUNG.T - tumour

M

MDSC – myeloid-derived suppressor cells

N

NF- κ B – kappa-light-chain-enhancer of activated B cells
NK – natural killer
NSCLC – non-small cell lung cancer
ntHI – nontypeable *Haemophilus influenzae*

O

OTU – outer taxonomic unit

P

PAMPs – pathogen-associated molecular patterns
PD-1 – programmed cell death protein 1
PD-L1 – programmed cell death protein 1 ligand
PRR – pattern recognition receptors

R

ROC – receiver operation characteristic
ROR γ t - Retinoic Acid-Related Orphan Receptor gamma t
ROS – reactive oxygen species

S

SCC – squamous cell carcinoma
SCFA – short-chain fatty acids
SCLC – small-cell lung cancer
SCT – supraglottic-characteristic taxa
sIgA – secretory immunoglobulin A
SPF – specific-pathogen free
ST – smaller tumour

Abbreviations

STAT3 – signal transducer and activator of transcription 3

T

TAM – tumour associated macrophage

Tc – cytotoxic CD8⁺ T lymphocytes

TGFβ – transforming growth factor β

Th – T helper lymphocyte

TIGIT – T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains

TLR – Toll-like receptor

TME – tumour microenvironment

TNFα – tumour necrosis factor α

Treg – regulatory helper T lymphocyte

V

VEGF – vascular endothelial growth factor

W

WHO – World Health Organisation

List of publications and communications

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Publications associated to thesis project

Bingula R, Filaire E, Molnar I, Delmas E, Berthon JY, Vasson MP, Bernalier-Donadille A, Filaire M (2019) Characterisation of microbiota in saliva, bronchoalveolar lavage fluid, non-malignant, peritumoural and tumour tissue in non-small cell lung cancer patients: cross-sectional clinical trial. Submitted to *BMC Respiratory Research* the 5th December 2019

Bingula R, Filaire E, Talvas J, Berthon JY, Vasson MP, Bernalier-Donadille A, Radosevic-Robin N, Filaire M (2019) Inverse abundance pattern of tumour and extratumoural lung microbiota between non-small cell lung cancer patients with or without metastatic lymph nodes: a cross-sectional clinical study. Submitted to *BMC Microbiome* the 25th September 2019

Bingula R, Filaire M, Radosevic-Robin N, Berthon JY, Bernalier-Donadille A, Vasson MP, Thivat E, Kwiatkowski F, Filaire E (2018) Characterisation of gut, lung, and upper airways microbiota in patients with non-small cell lung carcinoma: Study protocol for case-control observational trial. *Medicine* 97(50): e13676. doi: 10.1097/MD.00000000000013676 (*Annex 1*)

Bingula R, Filaire M, Radosevic-Robin N, Bey M, Berthon JY, Bernalier-Donadille A, Vasson MP, Filaire E (2017) Desired Turbulence? Gut-Lung Axis, Immunity, and Lung Cancer. *Journal of Oncology*. doi: 10.1155/2017/5035371 (*Annex 2*)

Communications associated to thesis project

Bingula R. Etude MICA: le microbiote, le système immunitaire et le cancer de poumon - l'idée, le concept et les premiers résultats. Animation scientifique UMR 1019 INRA-UCA (8th January 2019) (oral communication)

Bingula R. Le microbiote pulmonaire : pour l'étudier, il faut d'abord le « trouver ». Journées de l'Ecole Doctorale des Sciences de la Vie, Santé, Agronomie, Environnement (n°21, Clermont-Ferrand, 14th-15th June 2018) (oral communication)

List of publications and communications

Bingula R. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy: Sivan et al. 2015. Journal Club of INRA de Theix (5th April 2017) (oral communication)

Bingula R. Microbiota, nutrition and lung cancer. Journée d'unité de la nutrition humaine – flashposter « Ma Thèse en 180 seconds » (15th June 2016) (oral communication)

Other publications

Bingula R., Dupuis C, Pichon C, Berthon J-Y, Filaire M, Pigeon L, Filaire E (2016) The antitumour effects of betaine and/or C-phycoyanin on the lung cancer A549 cells in vitro and in vivo. *Journal of Oncology*: e8162952. doi: 10.1155/2016/8162952 (*Annex 3*)

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Introduction

According to the World Health Organisation (WHO), lung cancer (LC) is the world's first cause of death by cancer with approximately 1,761,000 registered deaths and 2,093,876 new cases in 2018 (International Agency for Research on Cancer 2019). It is classified into two main groups: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Even though SCLC has worse prognosis and very high lethality, it accounts for ~5% of LC cases. NSCLC is therefore more frequent, with prognosis dependent on underlying mutations, histological type and degree of aggressiveness (Molina et al. 2008). In NSCLC, the leading prognostic tool is tumour staging based on TNM classification, dividing tumours into different stages depending on their size (T), metastatic changes on lymph nodes (N) and distant metastasis (M) (Goldstraw et al. 2016). More advanced stages, especially if including metastatic changes, are associated with worse outcome and significantly shorter overall survival (Planchard et al. 2019).

So-called “escape” mutations were considered as the major way of cancer to evade host's immune response. This means that cancer cells accumulate various mutations due to high turnover, lack of tumour suppressors and exterior mutagenic factors (e.g. smoke, alcohol) until they become unrecognisable to host's immune system (Dunn et al. 2002). However recently, this exclusively intrinsic approach has begun to loosen up, and cancer research has adopted more of a systemic approach. Several factors are now recognised to influence cancer surveillance, such as nutrition, physical activity, life style (Molina et al. 2008), to the most recent one – microbiota. Microbiota represents a consortium of bacteria, fungi, viruses and protozoa (Marsland, Trompette, and Gollwitzer 2015), but is most often used to refer only to bacteria (in this manuscript as well). Except in the gastrointestinal system, as the most studied, microbiota resides on the skin and within other host's cavities (urogenital tract, oropharyngeal area, etc.). With the increasing recognition of functions performed by the gut microbiota and their crucial role for the host, the gut microbiota is frequently considered as a “forgotten organ”. With the bacterial mass of 2 kg, 1-10-fold more cells and 100-fold more genes than in human cells, this “organ” assures host's homeostasis by nutriment degradation, precursor and vitamin synthesis, but also by stimulation of the immune system (Bashiardes et al. 2017; Flint et al. 2012; García-Castillo et al. 2016; Kamada et al. 2013; Kau et al. 2011; Sender, Fuchs, and Milo 2016; Zeng et al. 2016). Gut microbiota was recognised as the underlying factor of immune system development in early age (Gensollen et al. 2016; Thaïss et al. 2014), and its dysbiosis was associated to several immune disorders, such as asthma (Kalliomäki and Isolauri 2003). It

Introduction

is now known that commensal bacterial cells constantly prime the immune system, maintaining the “stand by” state that will respond more readily to foreign antigens, but also maintaining the “cool-down” mechanisms that will prevent overreaction (Curotto de Lafaille, Lafaille, and Graça 2010; Mazmanian et al. 2005; Noverr and Huffnagle 2004). This feature of commensal microbiota was recognised in the cancer treatment, leading to emerging of “gut-lymph” theory (Samuelson, Welsh, and Shellito 2015). It suggests that commensal bacteria in the intestines could stimulate anti-tumour immunity not only locally, but also systemically. This could happen through migration of bacterial cells, their products or primed immune cells to distal location through the lymphatic system or circulation. At the distal location, e.g. tumour bed, these factors could either stimulate anti-tumour response (same as in the gut), or already primed immune cells could directly exert their anti-tumour effect. The basis of this theory lies in several studies in animal models, where animals raised in germ-free (GF) conditions or treated with antibiotics did not respond to classical chemotherapy with cyclophosphamide or neoadjuvant immune checkpoint inhibitor therapies (Daillère et al. 2016; Routy, Le Chatelier, et al. 2018; Sivan et al. 2015; Viaud et al. 2013). On the contrary, introduction of selected bacterial strains in these animals restored response to therapy and even had anti-tumour effect if administered without chemotherapy (Sivan et al. 2015). These studies opened a huge field of interest into oncomicrobiotics, bacteria that could be used as an anti-cancer drug and improve immune surveillance (Routy, Gopalakrishnan, et al. 2018).

The increased interest in the features of microbiota and interaction with the host led to establishment of the Human Microbiome Project (HMP), aiming to obtain a complete characterisation of the human microbiome by joint forces of the worldwide scientists. However, not all microbiota were initially included, as for example microbiota of the lungs (Proctor 2011), decreasing the general interest in its study. Another inconvenience to exploration of the lung microbiota has been the difficulty of its sampling due to techniques’ invasiveness. This based most of the studies on bronchoalveolar lavage fluid (BAL) obtained by the bronchoscopy, but with accompanying risk of contamination by the upper airways due to the passage of bronchoscope (Bassis et al. 2015; Beck, Young, and Huffnagle 2012; Charlson et al. 2011). So far, most of the studies investigating lung microbiota consider chronic-obstructive pulmonary disease (COPD) (Banerjee, Khair, and Honeybourne 2004; Einarsson et al. 2016; Erb-Downward et al. 2011; Moghaddam 2011; Sze et al. 2015), asthma (Gollwitzer and Marsland 2014; Huang et al. 2011), and cystic fibrosis (Fodor et al. 2012; Garg et al. 2017) (often routine bronchoscopy that simplifies sampling). But finally, the past few years were marked with the

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first studies on lung cancer microbiota and its association with environmental factors (Yu et al. 2016) or increased inflammation in the lower airways (Segal et al. 2016). Even though certain studies already evoked the “magical” influence of the gut microbiota on response to lung cancer (Schuijt et al. 2016), the scientific community warned that it is first necessary to study the effect of the local lung microbiota before being able to evoke the new-established “gut-lung” axis (Dickson and Cox 2017).

In an attempt to be the first to provide a more complete vision of the lung microbiota in lung cancer, its association to local and systemic immunity, and finally, with the gut microbiota, we performed a clinical trial in NSCLC patients with and without neoadjuvant chemotherapy before surgery. In the scope of this thesis, only a group without neoadjuvant therapy will be addressed.

Our work has provided the first results on composition of the lung microbiota in NSCLC patients based on samples with different origin (BAL, non-malignant tissue, peritumoural tissue, and tumour), their difference between upper and lower lobes, but also their connexion to local immune response and presence of metastatic lymph nodes.

We have shown that BAL microbiota consists of a unique microbiota and that the bias in sampling of either BAL or tissue microbiota has been justified. Next, we have shown that the varying characteristics between the three tissues are visible only when putting the analysis “into perspective”, such as the factor of tumour lobe’s location or presence of metastatic lymph nodes (LN). Moreover, we have shown that tumour and extratumoural tissues have inverse abundances of aerobic and anaerobic genera depending on the metastatic status of LN. Finally, we have shown that BAL is the only sample whose genera are associated with protumoural and antitumoural markers of both immune cell phenotypes in BAL and with tumour infiltrating lymphocytes.

The manuscript begins with bibliographic background organised in four parts. First part introduces the problematic of lung cancer (types, staging), its connection to local and systemic immune response as well as risk factors. Since gut microbiota is the most explored and characterised microbiota in the terms of host interactions and the basis of microbiota-against-cancer approach, second part provides the essential knowledge on this matter. The third part introduces lung microbiota and its so-far known characteristics and interaction with immune system. Final part discusses the gut-lung axis in the form of the published review. Following the bibliographic overview, current results are presented as articles that answer to hypotheses and objectives. The manuscript ends with general discussion of the results and perspectives.

Literature overview

1 Lung cancer

1.1 Epidemiology

Lung cancer (LC) is a leading cause of death by cancer worldwide, responsible for 1,761,007 or 18.4% deaths in 2018 according to the World Health Organisation (WHO) (Figure 1) (International Agency for Research on Cancer 2019). It is also the most frequent cancer in men and the third most frequent in women (International Agency for Research on Cancer (IARC) 2014). Its poor survival rates post-diagnosis are due to its late and very often accidental detection, when the success rate of clinical intervention is significantly reduced. In average, the 5-year relative survival rate for non-small cell lung cancer, as the most common type, (NSCLC) is about 60% (Goldstraw et al. 2016).

Estimated number of deaths in 2018, worldwide, all cancers, both sexes, all ages

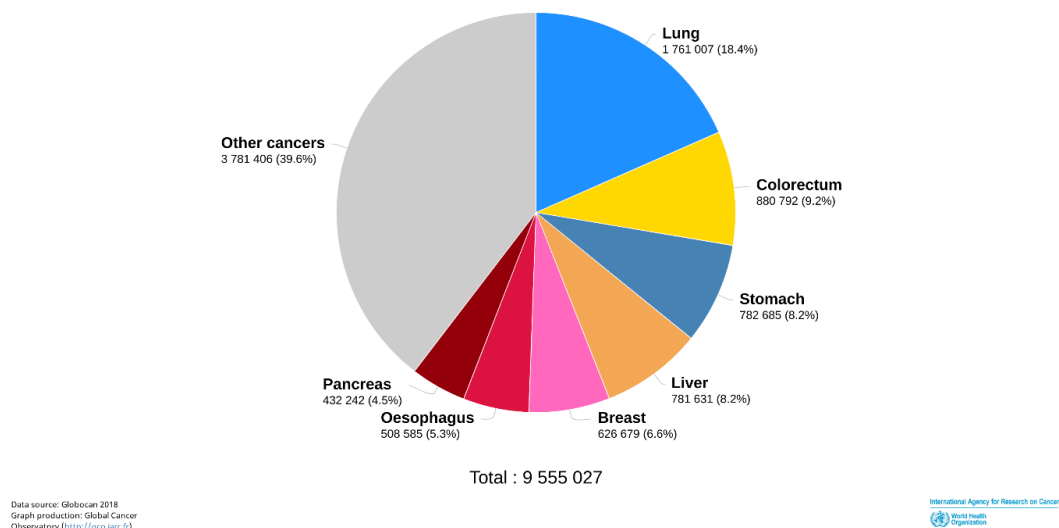


Figure 1 Estimated number of deaths in 2018 worldwide, all cancers, both sexes, all ages (adapted from International Agency for Research on Cancer 2019)

1.2 Lung cancer types

LC exists in many histological types (Figure 2), each linked to various aetiology, developmental patterns and prognoses (Mur et al. 2018). Three major groups of LC are small cell lung cancer (SCLC) (10-15% of lung cancers), NSCLC (80-85%) and lung carcinoid tumours (fewer than 5%)(American Cancer Society 2016). Since SCLC were not included in this study due to the dispersive nature of this type of cancer, the focus will be mostly on NSCLC.

There are three major types of non-small cell lung cancer:

- ❖ Adenocarcinoma
- ❖ Squamous cell lung carcinoma (epidermoid carcinoma)
- ❖ Large cell lung carcinoma
- ❖ Other (sarcomatoid carcinoma/salivary gland tumour/unclassified carcinomas)

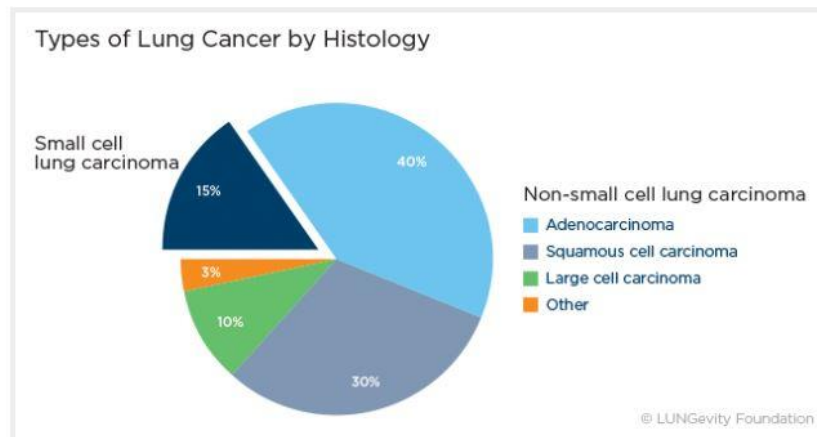


Figure 2 Prevalence of various histological types of lung cancer (adapted from LUNGeivity Foundation site)

1.2.1 Adenocarcinoma

Adenocarcinoma (ADK) accounts for about 40% of LC (Zappa and Mousa 2016) and is the most common histological type of NSCLC (Pallis and Syrigos 2013). It develops from early versions of “gland” cells (the name “adeno”), and can often (but not always) be distinguished from other tumour types by excessive mucus production at tumour site. It is often localised in the outer parts of the lung and tends to grow slower than other tumour types. It is more common in women than in men (Nagy-Mignotte et al. 2011), and is the most common type of lung cancer seen in non-smokers (American Cancer Society 2016; Nagy-Mignotte et al. 2011).

1.2.2 Squamous cell carcinoma (epidermoid carcinoma)

Squamous cell carcinoma (SCC) accounts for 25-30% of LC (Zappa and Mousa 2016). Unlike adenocarcinoma, SCC originates from early versions of squamous cells, forming the thin monolayer on the inside of the airways that separates alveolar lumen from blood vessels. These tumours are mostly located in the central part of the lungs, near the main bronchus. It is often found in men of age and the history of smoking is strongly related to its development. SCC is a slow-growing tumour that develops late metastasis, which makes it suitable for treatment by surgical resection (Popper 2016). However, this tumour type does not show high sensitivity to chemoradiotherapy (Xue et al. 2016).

1.2.3 Large cell carcinoma

Large cell carcinoma accounts for 5-10% of LC (Zappa and Mousa 2016). It is characterised by large, poorly differentiated cells that tend to grow and spread quickly. Certain subtypes can be similar to small cell LC, e.g. large cell neuroendocrine carcinoma. It can appear anywhere in the lung without site-specific affinity (American Cancer Society 2016).

1.3 Lung cancer staging

Staging system involves the assessment of cancer spreading by attributing different degrees to three main characteristics: size and extent of the main tumour (T), the spread to nearby lymph nodes (N), and the spread (metastasis) to distant sites (M). Therefore, the staging system is also called the TNM system, and is defined by the American Joint Committee on Cancer (Goldstraw et al. 2016).

Stages range from 0 to IV, 0 being the earliest stage (also called the carcinoma *in situ*). Likewise, within the stage an earlier letter/number designates a lower sub-stage. Interestingly, cancer stage seems to be more important than its proper type, since same stages of different cancer types show similar outlook and are often treated in the same way (American Cancer Society 2016; Novello et al. 2016). For example, 5-years survival rate following surgery is ~90% for stage IA1 as the least aggressive stage, and ~12% for stage IIIC as the more advanced (Asamura et al. 2008; Goldstraw et al. 2016).

1.4 Tumour and immunity

In 2001, with a profounded update in 2011, Hanahan and Weinberg (Hanahan and Weinberg 2011) presented an intuitive descriptive of a multistep initiation, transformation and progression of normal cells towards a tumorigenic profile under the name “Hallmarks of cancer” (Figure 3). Our immune system is designed to meet up to these changes by close surveillance of our body (by e.g. dendritic cells (DC)) that can elicit anti-tumour response (so called “elimination” phase). However, certain tumour cells are able to evade this “cleaning” phase, either by reduced surface recognition molecules or by producing immunosuppressive cytokines (Dunn et al. 2002). These surviving cells begin to expand in a dynamic equilibrium with the immune system; cells with genetic changes that result in recognisable epitopes are eliminated by the immune system, others continue to propagate (Bashiardes et al. 2017; Hanahan and Weinberg 2011). When tumour is no more recognised by immune system, it enters the “escape” phase (Dunn et

al. 2002) characterised also by its accentuated immunosuppressive nature: recruitment of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSC) to cancer site (Bashiardes et al. 2017).

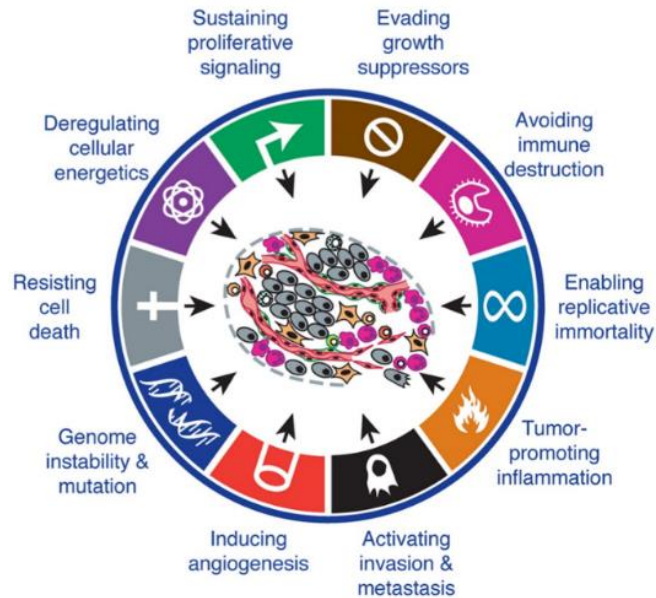


Figure 3 Hallmarks of cancer (adapted from Hanahan and Weinberg, 2011)

NSCLC also belongs to this group of tumours that create an immune-privileged microenvironment based on immunosuppression. The production of immunosuppressive cytokines by tumour cells induces an increase in the frequency and suppressive capacity of Tregs (Ju et al. 2009; Koyama et al. 2008), but also the expression of suppressive surface molecules. One such molecule is the programmed cell death-1 (PD-1) receptor on CD8⁺ T cells, which binds its programmed cell death protein 1 ligand (PD-L1) in the tumour bed (Yannelli et al. 2009; Zhang et al. 2010), leading to the abrogation of anti-tumour response. Furthermore, NSCLC tumour cells have a possibility to affect DC cells, “upstream” of the final response, by the inhibition of their maturation and hence the induction of the adequate immune reaction (Perrot et al. 2007; Tabarkiewicz et al. 2008). These DCs produce higher amounts of transforming growth factor β (TGF β) and are therefore strong inducers of Tregs (Dumitriu et al. 2009).

Interleukin (IL)-17 was recognised as another important factor in a progression of NSCLC. Secreted by CD4⁺ T helper (Th) Th17 cells, macrophages and CD8⁺ T cells (Rouvier et al. 1993), IL-17 is shown to stimulate production of, among others, IL-6, IL-8, IL-18, tumour necrosis factor α (TNF α) and vascular endothelial growth factor (VEGF) (Numasaki et al. 2003; Tartour et al. 1999). These cytokines favour Th2 immune profile that in tumour environment stimulates angiogenesis and tumour progression (Nam et al. 2008; Numasaki, Lotze, and Sasaki

2004; Wang et al. 2009). In addition, IL-6 has been identified to play essential role in lung cancer by promoting chronic obstructive pulmonary disease (COPD)-like inflammation (Ochoa et al. 2011). The role of IL-17 (and the whole Th17 set) in the lung tumour immunology was subject for a long and a complex debate, since several studies reported positive effect on effector cytotoxic T cell generation and induction of an anti-tumour response (Kryczek et al. 2009; Martin-Orozco et al. 2009; Muranski et al. 2008). However, the recent metastudy analysing the results of 6 lung cancer cohorts (total nb. of participants = 479) associated elevated IL-17 concentrations with significantly reduced overall and disease free survival, respectively, siding increased production of IL-17 and Th17 profile with negative effect on lung tumour progression (Wang et al. 2017).

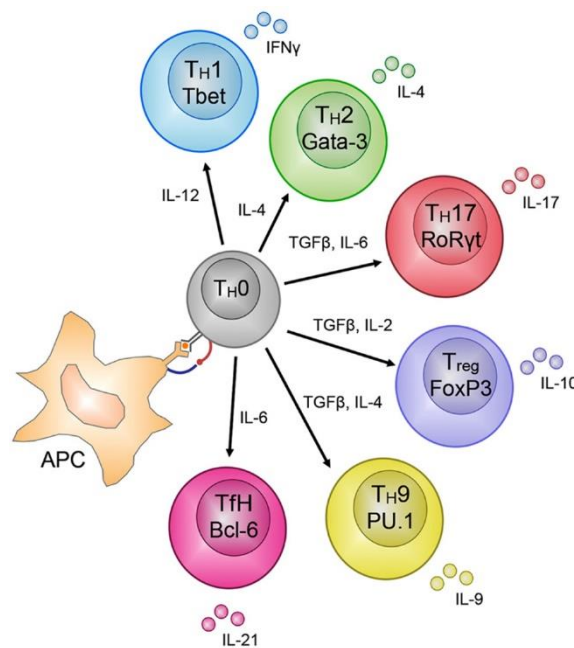


Figure 4 Overview of the basic Th profiles and their stimulating/producing cytokines (adapted from Russ et al. 2013). Naïve $CD4^+$ Th are stimulated by the antigen-presenting cells (APC) to differentiate into one of the six current mature Th profiles. Except the signal from the APC, differentiation requires environmental signals such as the presence of certain cytokines. Both stimulate expression of specific transcription factors responsible for differentiation and development of final Th subset with a set of its respective produced cytokines.

As a reminder, $CD4^+$ helper T cells are divided into different profiles dependent on the secreting cytokines and function: Th1, Th2, Th9, Th17, and Treg (Figure 4). Until recently, studies in lung cancer considered only the Th1/Th2 ratio as the major indicator of the balance between pro and antitumour response (Duan et al. 2014). Long-lasting dogma classified Th1 cells, producing IL-2 and interferon gamma ($IFN\gamma$), in antitumour response profile, while Th2 have been considered as a part of protumoural response, producing IL-4, IL-5, IL-6, IL-10, and IL-13. The balance between Th1 and Th2 in peripheral blood was shown to differ between healthy

controls and lung cancer patients (Ito et al. 2005). The increase of concentration of Th2 cytokines was suggested to shift balance into immunosuppressive profile (Becker 2006; Pinto et al. 2006), which as a consequence creates more tumorigenic environment (Green et al. 2010; Krohn et al. 2011) and higher relapse rate (Hong et al. 2013; Liang et al. 2011; Wei et al. 2003).

However, the ratio Th17/Treg started to increase attention, due to the reported tumour-influencing nature of these two cell sets (Marshall et al. 2016). First, it is important to say that both sets share common progenitor, expressing both retinoic acid-related orphan receptor gamma t (ROR γ t) and fork-head box protein 3 (FOXP3) genes, and chemokine receptors CCR6 and CCR4 (Duan et al. 2014). TGF β was found as a central element that differentiates the two cell types. The presence of IL-1 β , IL-6 and low dose of TGF β stimulates common progenitor to differentiate into Th17 cell set by stimulating the expression of ROR γ t genes. The differentiation itself is stimulated by DCs activated by microbes (Crome et al. 2009). On the other hand, the presence of retinoic acid stimulates the production of TGF β , and both have the potential to inhibit ROR γ t expression (i.e. Th17 development), favouring FOXP3 expression and differentiation towards Treg cell set (Peck and Mellins 2010). However, it has been reported that Tregs can convert to Th17 in the presence of inflammatory signals, such as IL-1 β , IL-6, IL-21, IL-23 (Duan et al. 2014).

Th17/Treg balance is particularly interesting in lung cancer due to overexpression of TGF β by lung cancer cells. Therefore, they favour Treg differentiation, associated with immune suppression and decreased anti-tumour response (Ju et al. 2009). This is also supported by the studies reporting that higher Th17/Treg ratio was negatively correlated with the tumour stages, and higher TGF β with the cases of metastases (Duan et al. 2014; Roberts and Wakefield 2003).

1.5 Risk factors for lung cancer

Even though genetic predisposition to mutations and consequentially development to lung cancer were described first, other factors like pollution, tobacco, alcohol, infections, hormones and immune competence of the host are now also recognized as factors that can increase the risk of lung cancer development (Plottel and Blaser 2011; Zong, Cao, and Wang 2012).

1.5.1 Smoking

Smoking is still recognized as the primary risk factor for lung cancer development. Its malefaction lies in the components of the smoke that act as DNA adducts, favouring carcinogenesis (Ahrendt et al. 2000; Lim et al. 2016; Nagy-Mignotte et al. 2011). Another

consequence of smoke particle inhalation and extensive heat production is the development of chronic inflammation that is thought to be an underlying cause of 25% of lung cancers (Balkwill and Mantovani 2012; Hanahan and Weinberg 2011; Mur et al. 2018; Walser et al. 2008). As reported in the study of Berger et al. (2016) on smokers without COPD, the elevated forced oscillation (as a measurement of respiratory function) was elevated in smokers and significantly associated with two-fold higher lymphocyte and neutrophil counts and higher pro-inflammatory markers (IL-8, eotaxin, fractalkine). This distal airway dysfunction lines up with similar observations reported in patients with established COPD and could add to understanding of higher risk of COPD development in smokers and its progression to cancer (Cho et al. 2011; Palucka and Coussens 2016).

1.5.2 Chronic inflammation

Although in general population smoking is considered, and presented, as one of the most influential factors to lung cancer development, it is interesting that only 10-15% of smokers develop cancer (but 90% of cancer cases consider smokers) (García-Castillo et al. 2016; Houghton 2013; Pevsner-Fischer et al. 2016). Chronic inflammation is considered to be a true culprit, already shown in the clinical research as the one increasing the risk of neoplasm (Greer and O'Keefe 2011; Louis, Hold, and Flint 2014). Proinflammatory cytokines in the tumour bed induce production of reactive oxygen species (ROS) by macrophages, which damage DNA, obstruct repair mechanisms, stimulate pro-tumorigenic pathways such as mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) transcription factor (Francescone, Hou, and Grivennikov 2014; Klaunig, Kamendulis, and Hocevar 2010). All this leads to the increased genetic instability (Elinav et al. 2013) and creates a favourable environment for tumour progression.

2 Microbiota and connection to lung cancer

2.1 What is microbiota?

Although most often used as a synonym for “bacteria”, “microbiota” is a much broader term that includes bacteria, archaea, fungi, viruses and protists that inhabit our organism in different types of commensal relationship (Hassan, El-khattouti, and Tandon 2013; Plottel and Blaser 2011). The gather of their genes is therefore called the “microbiome” (Clemente et al. 2012). Its impressive size that surpasses human genome up to 100 times and is closely related to host’s homeostasis is appropriately called the “second genome” or “the forgotten organ” (Arslan 2014; Dietert and Dietert 2015; Schwabe and Jobin 2013).

2.2 Gut microbiota – a role model

Gut microbiota is without a doubt the most extensively studied microbiota in humans. Containing more than 100 trillion microbes, it is one of the most complex and the most abundant ecosystems. In its balanced state, *Firmicutes* and *Bacteroidetes* are the two dominant phyla, while other less abundant are *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* (Belizário and Napolitano 2015). However, their balance can be gravely different between individuals, depending on their diet, life habits, origin, genetics, which has been already extensively studied (Figure 5) (Arumugam et al. 2011; Bäckhed et al. 2012; Eckburg et al. 2005; Segata et al. 2012; Zoetendal, Rajilic-Stojanovic, and de Vos 2008).

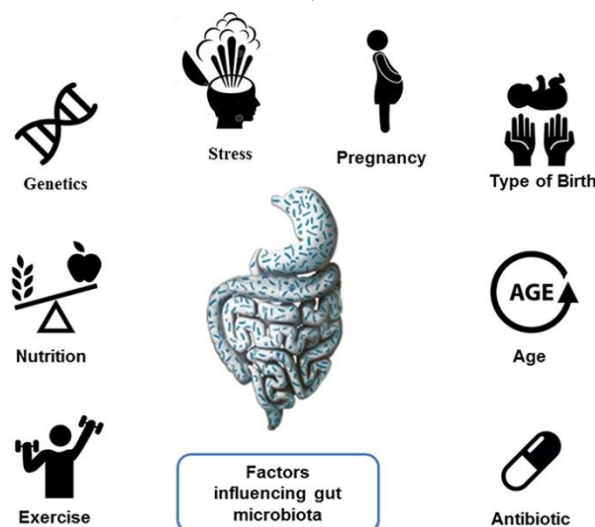


Figure 5 The factors with a direct influence on the gut microbiota (adapted from Cerdá et al. 2016)

2.2.1 Diet and immunity

Gut microbiota is known to have many different roles essential for its host's homeostasis; hydrolysis of dietary compounds, synthesis of vitamins, stimulation of immune system, control of pathogen colonization, development of intestinal barrier, and regulation of fat storage as only some of them (Bashiardes et al. 2017; Flint et al. 2012; García-Castillo et al. 2016; Kamada et al. 2013; Kau et al. 2011; Zeng et al. 2016).

Following the studies on the mucosal immunity (Fujimura et al. 2014; Noverr et al. 2004, 2005), gut microbiota was found to be responsible for the maturation of the overall immune system. In GF animals, the immune system was underdeveloped and with pronounced immunosuppressive character. This was however reversible by colonization with conventional microbiota (Chung et al. 2012). Even though certain genera or species were correlated to the production of cytokines, regulation of systemic inflammation or metabolic pathways, it was suggested that the microbial functionary groups sharing same metabolic roles could have much greater importance than e.g. genera with similar taxonomical classification (Blander et al. 2017; Schirmer et al. 2016).

Anaerobic fermentation of non-digestible polysaccharides, such as dietary fibre, is one of the most extensively studied roles of gut microbiota and also its importance in maintaining gut-host homeostasis (Zoetendal et al. 2008). Through fermentation and carbohydrate hydrolysis, colonic bacteria produce carbon dioxide, methane, hydrogen and short-chain fatty acids (SCFA) that can affect gut barrier stability, inflammation and gut hormone regulation (Slavin 2013).

Among fermentation products, it is important to emphasize the role of three SCFAs: acetate, propionate and butyrate. In human caecum, they are found in 70:20:10 ratio, respectively (Lloyd and Marsland 2017). They are produced in majority from the members in the genera *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Coprococcus*, *Methanobrevibacter*, and families *Clostridiaceae* and *Lactobacillaceae*. SCFAs, and especially butyrate, are used by colonocytes as energy source and thus are extremely important for the maintenance of the gut barrier (Meijer, de Vos, and Priebe 2010). Furtherly, they are shown to have anti-inflammatory properties, to inhibit biofilm formation and pathogens activity, and decrease tendency of type-2-diabetes development (Belizário and Napolitano 2015; Le Chatelier et al. 2013; Corrêa-Oliveira et al. 2016). SCFAs are also reported to influence function, maturation and fate of immune cells (Atarashi et al. 2013; Le Poul et al. 2003; Vinolo et al. 2011). They are ligands of surface receptors found on immune cells, G-protein coupled receptor 41 (GPR41), GPR43

and GPR109a (Maslowski et al. 2009; Smith et al. 2013; Trompette et al. 2014). Moreover, butyrate was found to be a very potent inhibitor of histone deacetylase, which stimulates maturation of Treg cells via expression of FOXP3 gene, exerting anti-inflammatory properties (Kim et al. 2007). Also, uniquely butyrate showed to inhibit proliferation of intestinal stem cells and progenitor cells during mucosal injury, which might prevent tumorigenic transformation under inflammatory conditions (Kaiko et al. 2016). Since butyrate is approximately used as 80% of energy source for colonocytes, it is not a surprise that it promotes colonic oxygen consumption by stabilizing transcription factor hypoxia-inducing factor 1 (HIF-1), which is responsible for transcription of a set of anti-tumorigenic genes (Kelly et al. 2015).

A variety of other bacterial metabolites are found to be similar to metabolites produced by human cells (Wikoff et al. 2009) and are thought to be one of the key components for microbiota-host interaction. This chemical mimicry, especially of signalling molecules, could represent one of the bases for future therapeutic usage (microbiome-biosynthetic gene therapy) (Cohen et al. 2017).

Gut microbiota has also been implied in the direct interaction with cancer (Figure 6), both in the preventive and promoting aspect. Those will be discussed in the following two chapters, with the accent on the proposed and established mechanisms.

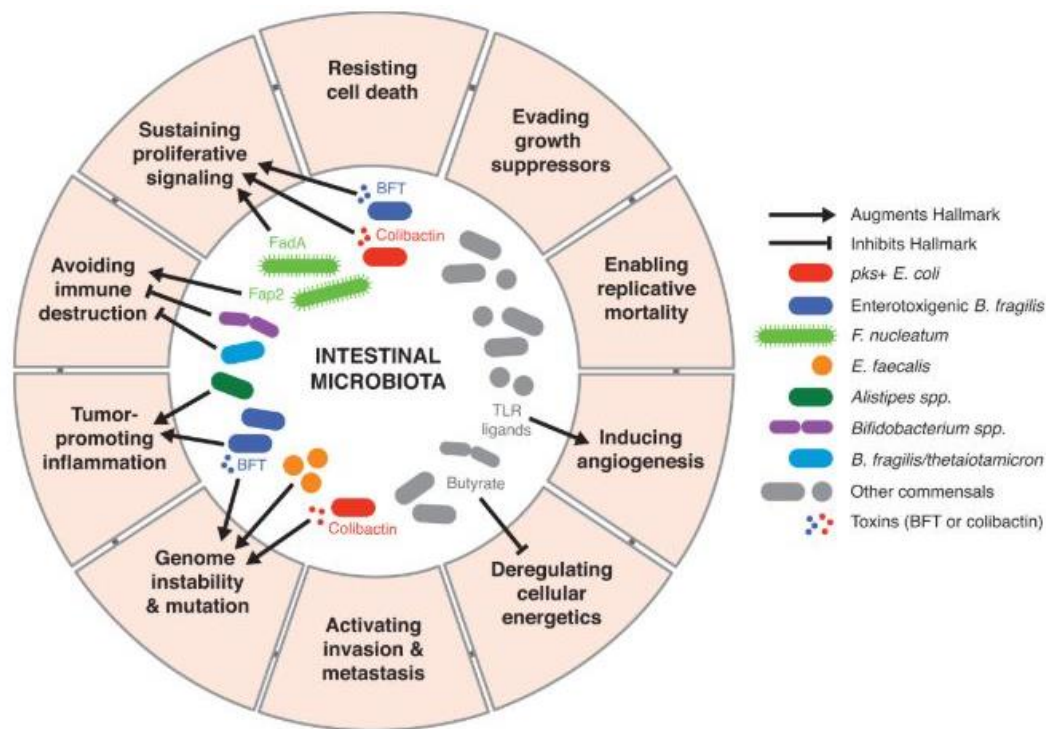


Figure 6 Modulation of hallmarks of cancer by microbial-derived signals (adapted from Fulbright, Ellermann, and Arthur 2017)

2.2.2 Gut microbiota for cancer

2.2.2.1 High-protein and unbalanced diet

Except fibres, high-protein diet also increases colonic fermentation (Louis et al. 2014). Fermentation of aromatic amino acids produces phenols, indoles, p-cresol and phenylacetic acid that could act as bioactive metabolites (Rajilić-Stojanović 2013; Schwabe and Jobin 2013). Ammonium, also a product of protein fermentation, has been proven to be carcinogenic in low concentration, as well as N-nitroso compounds inducing nitrogen alkylation of the DNA (Louis et al. 2014).

In anaerobic conditions, nitrate, sulphates and organic compounds replace oxygen as electron acceptor. Therefore, sulphate-reducing bacteria that compete for hydrogen have an important impact on cross-species interaction and changes in diversity. In normal population, they are found in low concentrations, but their higher abundance can be harmful (Louis et al. 2014; Rajilić-Stojanović 2013). The product of sulphate reduction is hydrogen sulphide, which inhibits oxidation of butyrate. It is toxic for colonocytes, proinflammatory, inhibits mucus production and is genotoxic (generation of free radicals) (Louis et al. 2014; Ridlon, Kang, and Hylemon 2006).

2.2.2.2 Disruption of barrier

Intestinal barrier with its mucosal layer, tight junctions between colonocytes, constant microbe monitoring by M-cells and secretion of multiple anti-microbial molecules represents a real anatomical separation with intestinal lumen (Schwabe and Jobin 2013). Its disruption leads to translocation of microbes or their products into systemic circulation and could trigger inflammatory response (Belizário and Napolitano 2015; Garrett 2015; Logan, Jacka, and Prescott 2016). The disruption could be caused by local inflammation, infection or defect genes (Garrett 2015; Schwabe and Jobin 2013). The deficiency of dietary fibres could also create a dysbiosis with proliferation of mucus-degrading bacteria. This showed to lead to thinning of the mucus layer with increased adhesion of the intestinal bacteria to colonocytes, and increased susceptibility to different pathogen colonisation, such as *Citrobacter* infection (Desai et al. 2016). In already established tumours, this secondary inflammation was shown to enhance intestinal tumour progression (Grivennikov et al. 2012; Huber et al. 2012) or increase a risk of its development in inflammatory bowel disease (IBD) patients (Ekobom 1991).

2.2.2.3 Microbial products

To find their place within this vast intestinal ecosystem, many microbes have developed various mechanisms that would give them selective advantage in competition and survival. One of these mechanisms are the DNA-damaging proteins, which cause genome instability and lead to mutations (Garrett 2015). There are many examples of bacteria with oncogenic potential, amongst others genotoxic *Escherichia coli*, *Enterococcus faecalis*, *Bacteroides fragilis*, *Fusobacterium nucleatum* (Arthur et al. 2012; Francescone et al. 2014; Kostic et al. 2013; Ohtani 2015; Schwabe and Jobin 2013; Sheflin, Whitney, and Weir 2014; Wu et al. 2009).

Fusobacterium nucleatum, a commensal from the oral cavity (Han and Wang 2013) already linked with certain pathogenic conditions (Lagier et al. 2012; Témoins et al. 2012), was recently connected to pathogenesis of colon adenocarcinoma. On its surface, *F. nucleatum* expresses adhesin FadA, which engages with E-cadherin on epithelial cells and stimulates their proliferation (Rubinstein et al. 2013), therefore is a potent stimulator of tumour cell growth. The other mechanism is realised through the effect on immune cells. Interaction of Fap2 protein of *F. nucleatum* with T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domains (TIGIT), which is an inhibitory receptor on natural killer (NK) and cytotoxic T (Tc) cells, inhibits tumour-killing properties of this two cell sets essential for identifying and destroying precancerous and malignant cells (Bashiardes et al. 2017; Gur et al. 2015). In animal models and clinical studies, *F. nucleatum* was also positively correlated to enrichment with tumour-associated macrophages (TAMs) and MDSCs, both with immunosuppressive and protumorigenic roles (Kostic et al. 2013).

2.2.2.4 Loss of diversity

High diversity of the gut microbiota is the virtue of a healthy, balanced intestinal environment. Loss of diversity was proposed to reflect the state of dysbiosis (Bäckhed et al. 2012) and was shown to be directly connected to inflammation, as seen in IBD, Crohn's disease and colorectal cancer (CRC) (Brown et al. 2013; Clemente et al. 2012; Fulbright et al. 2017; Sartor and Mazmanian 2012).

2.2.2.5 Neoangiogenesis

Gut microbiota was found essential for normal development of the intestinal vascular system (Stappenbeck, Hooper, and Gordon 2002). Interestingly, it was seen that during infection, toll-like receptors (TLRs) activated by bacterial lipopolysaccharide (LPS) promote angiogenesis, with the effect even more pronounced in the presence of damage-associated molecular patterns (DAMPs). Both are proposed to be a possible reason of microbial contribution to

neovascularisation seen in the tumour microenvironment, either directly by the colonising bacteria or pathogen-associated molecular patterns (PAMPs) and DAMPs within the microenvironment (Fulbright et al. 2017; Osheroov and Ben-Ami 2016).

2.2.2.6 Microbiota-induced Th17 inflammation

Th17 immune response is crucial in maintenance of the homeostasis of intestinal epithelium, and is driven by commensal microbiota and its products (reviewed in Bingula et al. 2017). However, it is also associated with protumorigenic activity and worse prognosis in CRC (Grivennikov et al. 2012). CRC murine models showed that colonisation with enterotoxigenic *Bacteroides fragilis* stimulates Th17-driven inflammation and cancer development (Housseau et al. 2016; Wu et al. 2009). Intestinal commensal *Alistipes* showed similar effect by enhancing the production of IL-6 and signal transducer and activator of transcription 3 (STAT3) activation (Moschen et al. 2016). Adversely, inhibition of IL-17 signalling axis reduced STAT3 promotion of inflammation and tumorigenesis (Housseau et al. 2016; Wu et al. 2009), confirming the application of Th17 signalling in CRC development and progression (Fulbright et al. 2017).

2.2.3 Gut microbiota against cancer

Over several decades, the cancer treatment gravitated towards more case-adapted and personalised approach. The currently used treatment includes surgery, radiotherapy, chemotherapy, and since recently immunotherapy and hormonal therapy (Bashiardes et al. 2017). Each of these is focused on “rectification” of immune response or eradication of the consequences of its failed activity. While some can truly bring instant salvation in certain cases (e.g. surgery), others are not always applicable or silently fail after first success, often leaving only palliative treatment. Furthermore, in certain cases the applied therapies have no effect whatsoever (e.g. chemotherapy, immunotherapy), leaving the physicians with no explication or solutions (Haslam and Prasad 2019; Prelaj et al. 2019).

Recently, certain studies started to search the answer in the giant ecosystem within our intestines – the gut microbiota. Even though as early as in 1920s, the intravesicular injection of bacillus Calmette-Guérin (derived from strain *Mycobacterium bovis*) into patients with superficial bladder cancer resulted in antitumour response and increased survival (Herr and Morales 2008), it was not until the 21st century that the answer to this irregularity in response to cancer therapy was connected and partially explained by our lifetime companions.

2.2.3.1 How does it work?

The proposed mechanism by which various microbiota elicit antitumour response lies in activation and stimulation of the immune system and possible similarity between bacterial and tumour epitopes, causing cross-reactivity (Zitvogel et al. 2016). LPS, flagellin, peptidoglycans or other microbial molecules that could serve as PAMPs are the ones behind priming and activation of the immune cells (Mogensen 2009; Ranf 2016).

Different bacteria could therefore indirectly induce antitumour response as seen in *Clostridia* spores found within solid tumours (Morrissey, O'Sullivan, and Tangney 2010). Those or alike could induce TNF α secretion, which vasoactive effect was shown to facilitate further entry of the bacteria into the tumour environment, led to CD8⁺ T cell activation and enhanced tumour surveillance (Leschner et al. 2009; Stern et al. 2015). Tumour colonisation by bacteria was also suggested as a result of the gut barrier disruption and bacterial translocation (Balzan et al. 2007).

Further, several studies in animal models investigated the role of balanced or dysbiotic gut microbiota on chemotherapy effectiveness, investigating the reason behind the effect of non-responding patients. Their results have opened a new area on importance of the gut microbiota – host relation through the interaction with the immune system, and have provided a new way of thinking in clinical therapeutic design. The following paragraphs summarise the most important discoveries of these studies.

2.2.3.2 Gut microbiota in chemotherapy

Chemotherapy is a non-selective cancer treatment, most often based on alkylating agents that act as DNA adducts, which are genotoxic and induce apoptosis in fast proliferating cells (tumour cells, but also some types of normal cells). Following apoptosis or necrosis, a liberation of tumour DNA and other internal proteins serves as immunogenic marker, stimulating T cell immunity and antitumour response (Alcindor and Beauger 2011). The following studies addressed the most commonly used drugs: platinum-based compound (still the most common in LC treatment) oxaliplatin, and cyclophosphamide (CTX) (Bracci et al. 2007). CTX was also shown to affect tumour environment by inducing reduction in Treg, and stimulating Th1 and Th17 (Routy, Le Chatelier, et al. 2018; Schiavoni et al. 2011).

The study of Iida et al. (2013) examined the effect of oxaliplatin on subcutaneous tumour (EL4 lymphoma, MC38 colon carcinoma and B16 melanoma) in different murine models. Conventional mice showed tumour regression and had improved survival, while in GF or antibiotic-treated mice this effect was much weaker. They found that TLR agonists, coming

from the commensal microbiota, promote ROS generation by the tumour-infiltrating myeloid cells causing cell death. Oxaliplatin alone functions on the same principle, but as explained, without microbiota the effect was more modest. Conventional mice with defect TLR signalling had the same response as GF/antibiotic-treated mice, and the other way around, GF mice administered with LPS showed a full response. The study also looked at the efficacy of the combination of intratumour CpG-oligonucleotides and anti-IL-10R antibody. Conventional mice showed retarded tumour growth and prolonged survival by rapid induction of intratumoral haemorrhagic necrosis dependent on TNF α , while this mechanism was significantly impaired in antibiotic-treated mice. In conventional mice, the therapy also induced higher production of IL-12 by tumour-infiltrating myeloid cells and IFN γ in tumour-infiltrating T and NK cells. These factors negatively correlated with the genus *Lactobacillus* and positively with Gram-negative bacteria (genera *Alistipes*, *Ruminococcus* etc.). In the genus *Lactobacillus*, there were *L. murinum*, *intestinalis* and *fermentum*, previously reported for their anti-inflammatory effects. Gavage of the mice with *L. fermentum* attenuated TNF production, while *Alistipes shahii* reconstituted TNF response in antibiotic-treated mice.

Viaud et al. (2013) evaluated the potential connection between gut microbiota and CTX in anti-tumour response stimulation. They demonstrated that treatment with CTX disrupted the intestinal barrier and induced a severe reduction within the *Firmicutes* phylum (*Clostridium* cluster XIVa, *Roseburia*, *Coprococcus* and unclassified *Lachnospiraceae*, facilitating the translocation of the certain Gram-positive bacteria into mesenteric lymph nodes and spleen. Several cultivated bacteria included *L. johnsonii*, *L. murinus* and *Enterococcus hirae*. These bacteria induced “priming” of specific subset of “pathogenic” Th17, normally responsible for the anti-bacterial response, and activation of Th1 memory cells that exerted antitumor responses. GF or antibiotic-treated mice did not show the priming effect of Th17 in the spleen and therefore had impaired antitumor responses, and adoptive transfer of mature Th17 cells only partially restored the initial antitumor effect.

Following this discovery, Daillère et al. (2016) investigated the effect of oral supplementation with these bacteria, positively correlating with productive antitumor response (Viaud et al. 2013), in sarcoma bearing mice treated with CTX. They found that *Enterococcus hirae* translocated from the small intestine to secondary lymph organs increased intratumour CD8/Treg ratio thus stimulating tumour eradication, failed by other species like *L. johnsonii* or other. Additionally, they identified another species from the *Porphyromonadaceae* family, *Barnesiella intestinihominis*, which accumulated in the colon, were able to induce

polyfunctional CD4⁺ Th1 and CD8⁺ Tc1 effector cells and stimulate infiltration of IFN γ -producing $\gamma\delta$ T cells into tumour. Both were addressed as potent “oncobiotics” that could ameliorate standard chemotherapeutical treatment.

2.2.3.3 Gut microbiota in immunotherapy

The big success in the understanding of the tumour – immune system interaction in cancer treatment were the antibodies that “unleashed” the suppressed immune system by inhibiting the immune checkpoints, to let the immune system fight the cancer on its own. The two currently used therapies include anti-PD-1/anti-PD-L1 antibodies, preventing inactivation of antitumor CD8⁺ cytotoxic T lymphocytes within the tumour, and anti-CTLA-4 (cytotoxic T lymphocyte antigen 4), which enables priming and activation of antitumor cells in lymph nodes (Brahmer and Pardoll 2013). Despite the initial overwhelmingness by this new approach, the percent of non-responders and developed resistance has been very high (~80%) as well as the number of autoimmune responses (Prelaj et al. 2019).

Sivan et al. (2015) looked at efficacy of anti-PD-L1 therapy on melanoma in genetically identical C57BL/6 mice coming from two different facilities. First, in two mice groups the spontaneous tumour growth was significantly different, but cohousing or faecal transfer eliminated this effect. Sequencing of faecal microbiota identified significantly different microbial population between two mice groups. 257 taxa were different between groups, but genus *Bifidobacterium* was the only significantly correlated with productive antitumor T cell responses. Secondly, they tested the effect of anti-PD-L1 therapy in both mice groups, with larger tumours (LT) and smaller tumours (ST). The group ST, naturally enriched with *Bifidobacterium*, completely inhibited tumour growth with therapy administration. Interestingly, the ST group without treatment had almost equal tumour size as the LT group with treatment. Further, the authors showed that in LT group either treatment with anti-PD-L1 or oral gavage with *Bifidobacterium* equally reduced tumour growth. Moreover, the combination of two had been significantly the most efficient, with the effect similar to ST group with only anti-PD-L1. The closer immunological analysis showed that the main effect was not exerted on effector cells, but on the more upstream level, i.e. DC priming.

Matson et al. (2018) looked at microbiota in stool of patients with metastatic melanoma before the anti-PD-1 therapy and correlated their clinical response with microbiota composition. They identified *Bifidobacterium longum*, *Collinsella aerofaciens* and *Enterococcus faecium* as more abundant in responders. The ratio of total nb. of “beneficial” and “non-beneficial” OTUs was calculated for each patient, and they showed that all patients with the ratio over 1.5 had

belonged to responders. Further, they showed that GF mice with stool transferred from responders had improved tumour surveillance mediated by elevated tumour antigen specific CD8⁺ T cell response and more efficient anti-PD-1 therapy. Conversely, in mice with the stool from non-responders the therapy was completely ineffective. Their findings reinforced previous studies and suggested a new, simple way of possible use of “beneficial”-“non-beneficial” bacteria ratio in prediction of PD-1 efficacy in treatment of metastasis.

Vétizou et al. (2015) addressed different type of immune checkpoint inhibitor, the therapy with anti-CTLA-4 antibodies, ipilimumab. They tested ipilimumab efficacy on established sarcoma in specific-pathogen free (SPF) and GF mice and observed that in GF mice therapy had no effect. Antibiotics administered to SPF mice however abrogated the ipilimumab efficacy, pointing to the responsibility of the gut microbiota. Ipilimumab induced inflammatory changes in the intestines, observed as increased cell death and proliferation of intestinal epithelial cells (IEC). Authors identified CD4⁺ and CD8⁺ T cells (including intraepithelial lymphocytes (IEL)) responsible for IEC apoptosis in the presence of microbial TLR agonists, and concluded that ipilimumab compromised the IEC-IEL balance. Ipilimumab also induced rapid underrepresentation of *Bacteroidales* and *Burkholderiales* and relative increase of *Clostridiales*. Further, oral gavage of antibiotic-treated SPF mice with qPCR identified species *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* (*Bf*) and *Burkholderia cepacia*, or combination of the last two recovered the response to anti-CTLA-4 antibody. In GF mice, *Bf* induced Th1 response in tumour-draining lymph nodes and promoted maturation of DCs, both restoring anti-CTLA-4 response. Moreover, these bacteria reconstituted intestinal barrier damaged by anti-CTLA-4 therapy in antibiotic-treated mice. This supported the fact that overall efficacy of therapy was highly dependent on microbiota composition, affecting IL-12 and IL-10 levels, reported the authors. To conclude, they also performed faecal transfer from patients before therapy and followed tumour growth in mice, which corresponded to patient's response to therapy.

2.3 Lung microbiota in sickness and health

2.3.1 Environment of the lower airways

Vocal cords have been considered as a limit between upper and lower airways, and even though they are characterised by continuous mucosal layer, these two parts are both anatomically and functionally very different (Kronenberger and Meyers 1994; Shaker and Hogan 2000; Wu and Segal 2017). Pulmonary epithelium is composed of one thin layer of squamous cells, among which there are ciliated columnar mucus-secreting goblet cells and cells secreting surfactants, mutually connected in tight junctions. In healthy lungs, mucus production is at low rate and form a protective layer enough to maintain homeostasis (disables microbial adherence to cells) (Radicioni et al. 2016). On their surface, cells have pattern recognition receptors (PRRs) such as TLR that serve to recognize PAMPs from viruses, bacteria, fungi, protozoa, and intracellular parasites (Lambrecht and Hammad 2012). As a response, the cells excrete multiple antimicrobial peptides such as surfactant protein A, lysozyme, lactoferrin, defensins, complement components (Iwasaki, Foxman, and Molony 2017; Rogan et al. 2006). Another role of the epithelium lies in cilia that with their synchronised movements facilitate the clearance of particulates towards upper airways, where they are terminally removed by coughing (Lloyd and Marsland 2017; Radicioni et al. 2016).

2.3.2 Lungs are not sterile

The biggest common worldwide project for the research of the microbiota, The Human Microbiome Project (HMP), included nares and oral cavity as the sites of the research interest, but interestingly, not the lower airways (Proctor 2011). Therefore, until today the knowledge about lung microbial composition and its interaction with the host's physiology and immune system remains scarcely explored (Huang et al. 2015; Segal et al. 2013, 2016; Sze et al. 2015). There have been also other inconveniences, as the difficulty of sampling, low biomass of the samples and consequentially, the high risk of contamination by upper airways while sampling (100 to 10,000 times less of 16S rRNA gene copies in lungs) (Bassis et al. 2015; Charlson et al. 2011). The three sample types are most commonly found across the studies of lung microbiota: tissue, bronchoalveolar lavage fluid (BAL) and sputum. The one the least ethically acceptable (so, the rarest) but representing the lowest contamination risk is the tissue, initially obtained from lung transplantation patients. Sputum is the sample with the highest contamination risk but the easiest to obtain. Therefore, the most studies are realised on BAL that represents an acceptable compromise; direct sampling of the lower airways lung microbiota

by lobe washing, but with bronchoscope passing through the airways (Bassis et al. 2015; Bernasconi et al. 2016; Dickson et al. 2016; Dickson, Martinez, and Huffnagle 2014; Hilty et al. 2010).

Lungs are an extremely voluminous hollow system of connected branching tubes with constant changes in gradients of oxygen and carbon dioxide tension. This creates rather selective pressure on microbial communities that might exist in lower airways (Lieberman et al. 2013). Under healthy conditions, there is a constant turnover of bacterial communities in the lower airways, as reported both by culture-dependant and independent techniques (Venkataraman et al. 2015). The theory of adapted island model suggests that the upper airways are the source of microbial diversity of lower airways, and that this diversity decreases with furthering from the central bronchus towards more distant lung parts. It was brought up by Dickson *et al.* (2015) in the study including 15 healthy volunteers whose microbiota was sampled by BAL in different lung parts. In these subjects, the BAL microbiota of the upper lobes resembled more the microbiota of the upper respiratory tract than the BAL microbiota from lower lobes. Further, the authors have suggested that lung microbiota in healthy subjects is more influenced by the rate of immigration and elimination than by the local growth conditions. Those, they say, have more importance in the cases of advanced lung diseases.

Lung microbiota by its composition was clearly distinct from other microbiota such as oral, nasal, skin, vagina or stool microbiota (Yu et al. 2016). Likewise, *Proteobacteria* was reported as the dominant phylum in the tissues with average relative abundance of 60%. Although lung is the highly aerated organ, the reports of co-occurring anaerobes and aerobes in culture-independent studies suggest that biofilms might play a very important role in the lower airways microbiota (Costerton et al. 1994; J W Costerton, Stewart, and Greenberg 1999) and also in potential pathogenicity as seen in the case of *Pseudomonas aeruginosa* (Whitchurch et al. 2002).

2.3.3 Resident lung immunity

Postnatal exposure to different air microbes by either inhalation or aspiration is irreplaceable for proper education and maturation of lung immune system. It is also important for proper structure development as seen in germ-free (GF) animals that had smaller lungs and decreased number of matured alveoli than conventional animals (Ho Man, de Steenhuijsen Piters, and Bogaert 2017).

As effective gas exchange is essential for human life, the maintenance of immune balance in the lung is of exceptional importance (Ho Man et al. 2017). This homeostasis is maintained by tissue resident cells that at the same time create tolerance and ensure rapid responses against invading pathogens. This includes members of both adaptive and innate immunity, such as alveolar macrophages, DCs, memory T lymphocytes but also Tregs and $\gamma\delta$ T cells (Lloyd and Marsland 2017). Following the encounter with antigen, the additional antigen-specific lymphocytes can be recruited to the lung through expression of lung-homing chemokine receptors 4 (CCR4) and CCR8 (on e.g. Th2 cells) (Cho et al. 2016; Lloyd et al. 2000).

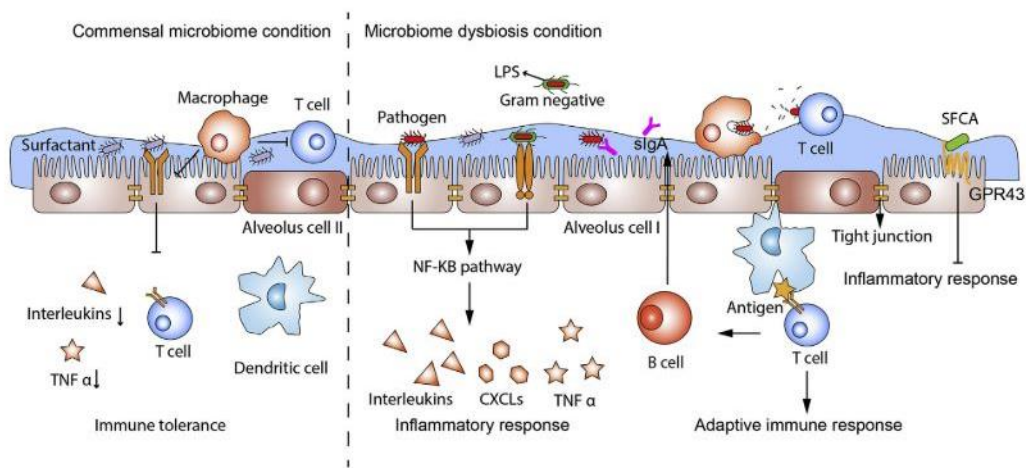


Figure 7 Homeostasis and dysbiosis in the lung epithelium (adapted from Mao et al. 2018).

Proposed homeostasis (left part of the figure) includes dynamic interplay between the commensal microbiota and local immunity (alveolar macrophage, lung DCs, several lymphocytic subtypes), maintaining a state of tolerance by decreasing inflammation and controlling local bacterial population through sIgA. A non-commensal microbiota introduces dysbiotic state, activating the PRRs and the NF- κ B cascade, inducing inflammation. Here, SCFA produced by local bacteria or delivered through circulation (produced e.g. in the gut) might have an anti-inflammatory role. CXCLs – chemokine (C-X-C motif) ligands, DC – dendritic cell, GPR43 – G-protein coupled receptor 43, LPS –lipopolysaccharide, SCFA – short-chain fatty acid, TNF α – tumour necrosis factor α

Commensal airway microbiota stimulates production of secretory immunoglobulin A (sIgA) that maintains constant dynamic balance between eliminated and colonised microbiota. Its deficiency leads to increased inflammation against commensal microbiota that could induce tissue damage (Richmond et al. 2016). Commensal microbiota is also directly responsible for phenotype of certain immune cells. For example, the stimulation of PD-1/PD-L1 expression by microbiota is directly connected to formation of inducible Tregs (Gollwitzer et al. 2014). These cells reside in the lung since birth, and are crucial in the maintenance of lung immune homeostasis (Lloyd and Hawrylowicz 2009). They are defined as CD4⁺CD25⁺FOXP3⁺ cells, and even though their major secretion product is immunosuppressive IL-10, their phenotype is influenced by the local cytokine milieu and infection status (Curotto de Lafaille et al. 2010; Krishnamoorthy et al. 2012; Morita et al. 2015). In allergy, their production of IL-10 is

responsible for amelioration of symptoms due to their immunosuppressive effect (Kearley et al. 2005; Lewkowich et al. 2005). However, in the case of the local infection by respiratory syncytial virus, they were reported to express GATA binding protein 3 (GATA3) gene (marker of Th2) and to produce Th2 cytokines. Therefore, instead of their immunosuppressive activity, Tregs contributed to Th2 mediated response and pathology in asthma (Krishnamoorthy et al. 2012). All this characterises lung homeostasis as a fine tuned environment of delicate equilibrium between resident microbiota and immunity (Figure 7).

2.3.4 Infection and inflammation

In health, lungs are a poor source of nutrients and therefore harbour rather low number of bacteria (Dickson et al. 2016). However, when our natural barriers, described earlier, do not suffice to eliminate potentially harmful particles or microbes, we talk about infection. Infection is accompanied by inflammatory response that, even though it clears its cause, leaves the tissue in the changed state by influencing local gas tensions, temperature or pH and could, therefore, create more favourable growth conditions for various bacteria (Dickson, Erb-Downward, and Huffnagle 2014; Mur et al. 2018). For example, mucus production is a common co-occurrence of inflammation, serving as a potential nutrient source (Dickson et al. 2016).

Furthermore, the immune system finds itself in immunocompromised state, meaning that surface markers for PAMP recognition (such as TLRs on e.g. macrophages or epithelial cells) or different genetic modification within cells might not return immediately to the same expression level as before the infection (Didierlaurent et al. 2008; Netea et al. 2016). This leads to the higher susceptibility of the lungs for recurrent infection. To illustrate, in the mouse model of COPD (model of inflammatory lung state that could mimic post-infection), exposure to LPS (mimics a new infection by bacteria) induced tissue damage and further altered microbiome causing diversity decrease (Yadava et al. 2016). Another example considers *Mycobacterium tuberculosis*, the pathogen responsible for pulmonary tuberculosis. The studies showed that persistent infection induced the production of TNF α and IFN γ , leading to inflammation and pulmonary fibrosis (Dheda et al. 2005). Furthermore, in certain cases lung cancer developed from this infection site “scars”, identifying the infection with *M. tuberculosis* as a risk factor for the development of lung cancer (Cukic 2017; Pallis and Syrigos 2013).

Although having a curative objective, the administration of antibiotics can also have a potentially undesirable effect on the community balance and infection. Its disturbance of the microbial communities, while clearing the target organisms, can lead to propagation of certain

surviving commensals that could become opportunistic pathogens or can clear a niche for pathogens already residing in the lung. Both ends up in the same process of infection and induces inflammatory response (Lloyd and Marsland 2017). Hospital pneumonia lies on this mechanism (Rello et al. 2009).

2.3.5 Age

Although at the moment very little is known about how does immune senescence affect lung responses, it is certain that lung habitat changes with age (Marsland and Gollwitzer 2014). The lungs become more susceptible to infections and inflammatory responses, as shown by the studies on COPD or pulmonary fibrosis (Lloyd and Marsland 2017). The role of the resident lung microbiota, as well as its “aging”, remains unknown for the moment.

2.3.6 Vices: smoking and alcohol

Previously, both alcohol and smoking have been acknowledged as strong risk factors for head and neck cancer (Vogtmann and Goedert 2016), as well as their synergistic effect for increasing the risk of lung cancer (Ahrendt et al. 2000). However, little is known about their interaction with lung microbiota.

Interestingly, even though smoking stays one of the main risks for lung cancer development, at the moment it seems that it does not directly influence the microbiota of the lower airways (Morris et al. 2013; Segal et al. 2013), unlike the one of the upper airways (Charlson et al. 2010; Wu et al. 2016). Nevertheless, more research is necessary, and future studies might identify smoking as a cofactor that will influence the disease and consequentially, microbial composition, rather than the cause of this modification.

Another vice causing local damage and inflammation is alcohol. The malice lies in the enzyme alcohol dehydrogenase, which converts alcohol to carcinogen acetaldehyde (Ahrendt et al. 2000). This enzyme and its high activity were noted in several microbial groups, amongst other in *Neisseria*, bacterial genus from the class *Betaproteobacteria*, a commensal found in oral mucosa (Muto et al. 2000). Therefore, high alcohol consumption could lead to overproduction of this and other carcinogenic metabolites that could descend into the lung and induce tissue damage and subclinical inflammation.

2.3.7 Microaspiration and pneumotypes

Microaspiration occurs in all individuals but is elevated in certain diseases such as COPD, asthma or lung infections, inducing the subclinical lung inflammation (Cvejic et al. 2011;

Huang et al. 2015; Koh et al. 2007; Morse et al. 2004; Segal et al. 2013; Segal, Rom, and Weiden 2014; Sze et al. 2012, 2015; Sze, Hogg, and Sin 2014; Teramoto et al. 1999). It has been suggested that the cause of inflammation lies in the enrichment of the lower airways with supraglottic taxa. In the study of BAL microbiota from 29 asymptomatic healthy subjects, Segal *et al.* (2013) detected two distinct pneumotypes. Pneumotype BPT (background predominant taxa) was characterised by low community richness and rRNA gene concentration, and associated with decreased production of proinflammatory cytokines, as TNF α , IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF). On the other hand, pneumotype SCT (supraglottic-characteristic taxa) had higher relative abundance of SCT such as *Veillonella* and *Prevotella*, significantly higher lymphocyte and neutrophil count and produced NO quantities. Other studies found similar results, with addition of *Rothia*, *Streptococcus*, and *Porphyromonas* to SCT pneumotype (Erb-Downward et al. 2011; Morris et al. 2013; Segal et al. 2013, 2016; Sze et al. 2012).

2.3.8 Chronic obstructive pulmonary disease (COPD)

COPD is a collective term for multisystemic inflammatory state that includes chronic bronchitis and emphysema (airway obstruction with disappearance of small airway sacs in the peripheral regions) (Houghton 2013). In 2016, there were 251 million cases worldwide with 3.17 million deaths the year before (WHO 2019). The underlying factor for COPD development in the western world is smoking, but also pollution and exposure to various particles.

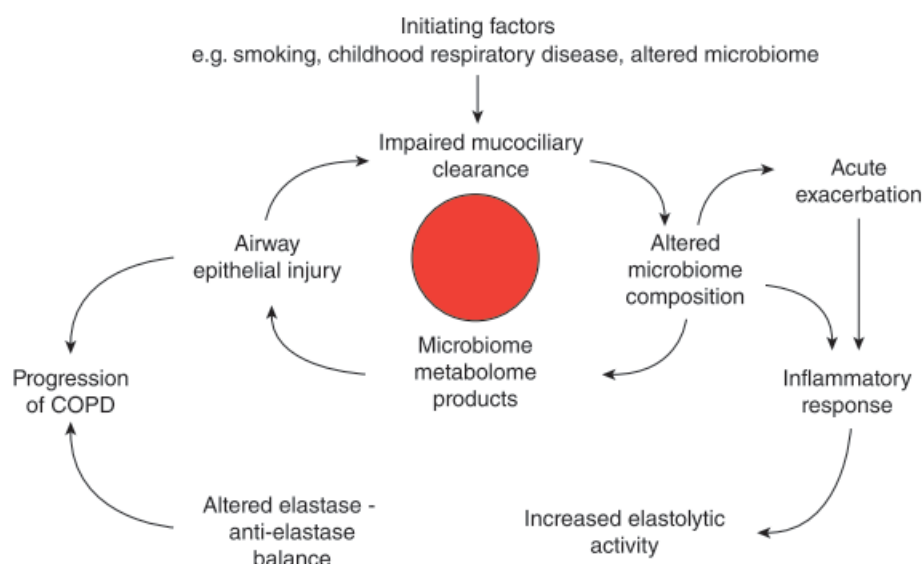


Figure 8 Vicious circle hypothesis in chronic obstructive pulmonary disease (adapted from Sethi and Mammen 2017)

Unlike smoking (Morris et al. 2013; Segal et al. 2013), COPD exerts distinct changes on microbial composition of the lower airways. One of the reasons is certainly its effect on

underlying lung architecture and activity of mucociliary surfaces (Wu and Segal 2017). As discussed in 2.3.4 Infection and inflammation, airways obstruction with increased mucus production could introduce pockets of increased temperature and decreased oxygen tension, which selectively favour the growth of microbes (Schmidt et al. 2014; Worlitzsch et al. 2002). Although results between studies tend to be inconsistent due to different sampling methods (tissue, BAL, sputum) (Sze et al. 2014), there is a consensus that acute exacerbations (episodes of crisis that worsen patient's state) are in 75% of cases connected to viral or bacterial infection, or a combination (Han et al. 2012). This intertwined cause-consequence relation between inflammation and bacterial colonisation has been explained by the vicious cycle hypothesis (Figure 8) (Mammen and Sethi 2017). Culture-dependent analyses have identified some of these bacteria that are already known as pathogens: *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* (Hirschmann 2000). Culture-independent studies based on BAL samples found that lungs of COPD patients were enriched by genera *Pseudomonas*, *Corynebacterium*, *Prevotella*, *Staphylococcus*, *Streptococcus*, *Veillonella*, *Haemophilus* and *Neisseria* (Erb-Downward et al. 2011; Sze et al. 2014). Sputum also showed to be indicative, since COPD severity correlated with differences in abundance of *Streptococcus pneumoniae*. The difference was also significant in the overall genus *Streptococcus* when comparing healthy subjects and ones with COPD (Cameron et al. 2016).

2.3.8.1 Chronic inflammation to lung cancer

Even though a role of inflammation in lung cancer does not naturally come first to mind, the development of lung cancer is closely related to chronic inflammation. In the situation of lung tissue injury or recognition of the foreign epitopes, there is an infiltration of inflammatory cells (Schmidt et al. 2014). However, for one reason or another, there is no resolution of inflammation, and the prolonged excretion of proinflammatory factors in the end leads to excessive tissue destruction. In the same time, the factors responsible for tissue remediation, as cytokines and chemokines that stimulate cell proliferation, angiogenesis and tissue remodelling in response to tissue injury, send survival signals to damaged or deregulated cells. All this creates a perfect environment for mutagenesis and metastasis (Cho et al. 2011; Palucka and Coussens 2016). Several studies already established the link between COPD and lung cancer development (Houghton 2013; Melkamu et al. 2013). Therefore, the role of lung microbiota in lung cancer was suggested as one of the important issues for consideration (Mur et al. 2018).

2.3.9 Lung cancer and microbiota

2.3.9.1 Egg or chicken

The question is simple – is the change in lung microbiota the cause of lung cancer development, or does the lung cancer development cause the change in surrounding microbiota. The answer is less simple, and it will probably rest highly dependent on each case (Blumberg and Powrie 2012; Francescone et al. 2014).

The colonisation of tumours by bacteria has already been suggested (Chanudet et al. 2007; Hooper et al. 2007). Growing, solid tumours stimulate neo-angiogenesis that results in leaky and irregular organisation, possibly enabling bacterial entry into the tumour to stimulate immune response (Bashiardes et al. 2017). However, in the same time the defective blood supply compromises drug delivery (Dang et al. 2002) and in itself is also not sufficient for oxygen needs of rapidly proliferating tumour cells (Vaupel, Mayer, and Höckel 2004). This leads to the creation of necrotic and hypoxic environment in the centre with increased activity of lactate dehydrogenase, which lowers pH by lactate production (Wouters et al. 2003). These particular conditions might furtherly favour colonisation and replication of anaerobic acidophilic bacteria (Baban et al. 2010). In addition, genetic transformation of tumour cells might stimulate the expression of certain epitopes that could serve as bacterial adhesion points not present in normal cells (García-Castillo et al. 2016; Garrett 2015; Khan, Shrivastava, and Khurshid 2012), facilitating tumour colonisation.

Both negative effect of this colonisation with direct role in carcinogenesis have already been well documented in other sites (e.g. *Helicobacter pylori*, *Fusobacterium nucleatum*) (Gur et al. 2015; Wroblewski, Peek, and Wilson 2010) and in different models (Schwabe and Jobin 2013; Sears and Garrett 2014), as well as the immune-stimulating properties of e.g. anaerobic *Clostridium* spores, enhancing tumour surveillance (Van Dessel, Swofford, and Forbes 2015). However, for the moment the involvement of lung bacteria in lung cancer development or suppression remains a mystery.

2.3.9.2 Microbiota in lung cancer

Until today, microbiota in lung cancer patients has been poorly studied. Unlike in asthma or COPD, routine bronchoscopy that could give direct possibility of microbiota assessment is not a common practice. Moreover, as discussed in the chapter 2.3.8.1 Chronic inflammation to lung cancer, until recently lung cancer was not associated with an inflammatory state nor was it treated as an outcome of infection with specific pathogen, therefore no true need was seen to

examine the microbiota within the tumour, or in the tumour environment. Similarly, the sampling methods that remain invasive and therefore difficult to realise (lung tissue or tumour samples) contributed to small number of studies of this type (discussed in the chapter 2.3.2 Lungs are not sterile).

Table 1 The studies identifying a significant difference in abundance of lung microbiota in lung cancer (adapted from Mao et al., 2018). ↑: Microbiota increases in LC compared to controls, ↓: Microbiota decrease in LC compared to controls.

References	Differential taxa features	Sample size	Sample type
Laroumagne et al. [65]	Genera: <i>Haemophilus influenzae</i> ↑ <i>Enterobacter</i> sp. ↑ <i>Escherichia coli</i> ↑	216	bronchoscopy
Hosgood et al. [15]	Genera: <i>Granulicatella</i> ↑ <i>Abiotrophia</i> ↑ <i>Streptococcus</i> ↓	16	sputum saliver
Yan et al. [66]	Genera: <i>Capnocytophaga</i> ↑ <i>Selenomonas</i> ↑ <i>Veillonella</i> ↑ <i>Neisseria</i> ↓ <i>Streptococcus</i> ↓	30	saliver
Cameron et al. [67]	Genera: <i>Granulicatella adicens</i> ↑ <i>Mycobacterium tuberculosis</i> ↑ <i>Streptococcus</i> ↓ Genera: <i>Escherichia coli</i> ↓ <i>Fusobacterium nucleatum</i> ↓	10	sputum
Lee et al. [68]	Genera: <i>Veillonella</i> ↑ <i>Megasphaera</i> ↑	28	broncho alveolar lavage
Yu et al. [13]	Genera: <i>Thermus</i> ↑ <i>Ralstonia</i> ↑	165	lung tissues
Liu et al. [69]	Genera: <i>Streptococcus</i> ↑ <i>Staphylococcus</i> ↓	42	lung tissues and bronchoscopy

The studies performed on microbiota from BAL remain the most common (Table 1). Even though for the moment without a direct connection with the disease, certain pathogenic bacteria belonging to Gram-negative bacilli (*H. influenzae*, *Enterobacter* sp., *Escherichia coli*) and Gram-positive *Mycobacteria* were identified in LC patients (Laroumagne et al. 2011). Another study compared BAL microbiota between LC patients and patients with benign biomasses as controls (Lee et al. 2016). They identified two phyla, *Firmicutes* and *TM7*, and two genera, *Veillonella* and *Megasphaera*, as more abundant in LC patients. The authors showed that these two genera together can predict LC with accuracy of 0.888. What is interesting is that *TM7* was also reported as elevated in certain studies on COPD, making it a potential microbial candidate for transition of COPD to LC (Mao et al. 2018). The authors also observed that the ratio between *Firmicutes* and *Bacteroidetes* was significantly higher in smokers than in non-smokers, suggesting that smoking might be the cofactor of microbial modification during the already established disease. Comparing BAL microbiota between the tumour lobe and contralateral lobe, Liu et al. (2018) noted significant decrease in diversity in the tumour lobe, as well as increased abundance of genera *Streptococcus* and *Neisseria*.

At the moment, only two studies have analysed microbiota of the non-malignant tissue paired tumour (Peters et al. 2019; Yu et al. 2016). Yu et al. (2016) defined the core microbiota found in 80% of non-malignant tissue samples as members of the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. Interestingly, at the genus level, five genera determined as core microbiota belonged to the phylum *Proteobacteria*: *Acinetobacter*, *Pseudomonas*, *Ralstonia* and two other from families *Comamonadaceae* and *Oxalobacteraceae*. When comparing with tumour tissue, the observed alpha diversity in non-malignant samples was

higher than in tumour tissue. The analysis by tumour histology indicated that adenocarcinoma had significantly increased phylogenetic diversity compared to squamous cell carcinoma, the increase in abundance of genus *Thermus* and the decrease in *Ralstonia*. Interestingly, Peters et al. (2019) associated higher diversity in non-malignant tissue with decreased recurrence-free and disease-free survival, while no link was seen with tumour microbiota.

Lung microbiota was also analysed in sputum samples of healthy subjects and LC patients. LC patients showed significant decrease in the abundance of phyla *Synergistetes* and *Spirochaetes* compared to healthy controls (Hosgood et al. 2015). Another retrospective study identified *Streptococcus viridis* as the only significantly more abundant in patients who developed lung cancer (Cameron et al. 2017)

In 2015, a paper was published on salivary microbiota demonstrating that LC patients had the increased abundances of genera *Capnocytophaga*, *Selenomonas*, *Veillonella* and *Neisseria* compared to healthy controls. Results on *Capnocytophaga* and *Veillonella* were further confirmed by qPCR. Moreover, the combination of these two genera yielded a receiver operation characteristic (ROC) value of 0.86 and 0.80 in distinguishing healthy subjects from patients with squamous cell and adenocarcinoma, respectively.

Regardless of the sample source, certain bacterial genera appear significant in several studies, such as *Granulicatella*, *Streptococcus* and *Veillonella* (Cameron et al. 2017; Hosgood et al. 2015; Lee et al. 2016; Liu et al. 2018; Yan et al. 2015). In addition, genera *Streptococcus* and *Granulicatella* are facultative anaerobes, while *Veillonella* is an obligate anaerobe (Mur et al. 2018), which could suggest the existence of biofilms and their potential role in LC. These findings confirm the hypothesis that lung cancer is accompanied by the specific changes in local microbiota. However, further studies are necessary to clarify this interplay and also to better characterise the changes depending on the location of sampling (tumour, adjacent tissue, non-malignant tissue, alveolar lumen, sputum, saliva).

2.3.9.3 Lung microbiota, Th17 and tumorigenesis

The ambiguous role of IL-17 was already addressed in the section “Tumour immunology” from the immune aspect. When observing its relation with microbiota, the implication in both protumoral inflammation and antitumoral response were described and were depending on the models of lung cancer (Chang et al. 2014; Cheng et al. 2014; Jin et al. 2019; Segal et al. 2016)

It was already evidenced in healthy subjects that the presence of supraglottic taxa in the lower airways was associated to subclinical inflammation marked by higher presence of Th17 phenotype and upregulation of IL-17 (Segal et al. 2016).

In COPD patients, nontypeable *Haemophilus influenzae* (nHI) induced secretion of epithelial IL-17C that increased neutrophilic infiltration and inflammation, promoting tumorigenesis (Jungnickel et al. 2017). Same authors demonstrated that exposure to combination of smoking and nHI induced translocation of bacterial factors that promoted proliferation of LC and metastatic growth (Jungnickel et al. 2015). Similarly but this time directly with cytokine producers, another study showed that nHI-induced Th17 cells could promote angiogenesis and LC cell proliferation (Chang et al. 2014).

Study of Jin *et al.* (Jin et al. 2019) investigated the interaction between LC and host microbiota using the mouse model with activated *KRAS* (Kirsten ras oncogene homolog) gene expression and loss of *P53* gene to simulate mutation frequently associated with ADK. They showed that local commensal microbiota stimulated production of IL-1b and IL-23 from myeloid cells, which induced proliferation and activation of $\gamma\delta$ T cells and their secretion of inflammatory cytokines including IL-17, to promote tumour inflammation and progression. Adversely, SPF mice treated with antibiotics showed suppressed tumour growth in both early and late stages. Likewise, antibody neutralisation of IL-17A significantly decreased neutrophil influx, tumour growth and IL-1b expression.

Completely opposite results were seen in the study of Cheng *et al.* (Cheng et al. 2014) on mouse model, where intact commensal microbiota were responsible for $\gamma\delta$ T cell response against LC. Mice treated with antibiotics were more prone to develop both B16/F10 melanoma and Lewis lung carcinoma, with more numerous and larger tumour foci in the lungs, and showing a defective $\gamma\delta$ T cell response. Transfer of normal $\gamma\delta$ T cells or injection of IL-17 rectified the aberrant antitumour response.

3 Review: The gut-lung axis and lung cancer

Review Article

Desired turbulence? Gut-lung axis, immunity and lung cancer

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The importance of gut microbiota in modulating host's immune and cancer response (see chapter 2.2.3 Gut microbiota against cancer) yielded in extensive research of communication between gut microbiota and different organs, called axes. Recently, a new axis has been proposed, named the “gut-lung axis”. Based on multiple indices that modification of a diet or exposure to certain particles changes the immune response in the lungs, and *vice versa*, the axis theory proposed the communication between these two compartments through lymphatic and blood circulation.

To approach these two very different microbiota, discuss their interaction with and through the immune system, and address its potential application in lung cancer, we wrote a review article entitled “Desired Turbulence? Gut-Lung Axis, Immunity, and Lung Cancer”. Its objective was to provide an overview of the current knowledge of the matter, which would then serve to guide the thesis research in the most pertinent direction for the scientific community.

The review addressed the current knowledge in the composition of the gut, rhino-oropharyngeal and lung microbiota in healthy subjects, and reported changes in COPD and lung cancer for lung microbiota. Next, it addressed the interaction between the mucosal immune system and the local microbiota, taking into account the influence of both bacterial cells and their products. Finally, the review considered the bidirectional concept of the gut-lung axis in the context of cancer, based on previously proposed lymph theory and the theory of the cancer-immunity cycle.

Review Article

Title: Desired turbulence? Gut-lung axis, immunity and lung cancer

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Conflict of Interest

The authors hereby declare that no conflict of interest exists with respect to the publication of this article.

Abstract

The microbiota includes different microorganisms consisting of bacteria, fungi, viruses and protozoa distributed over many human body surfaces including the skin, vagina, gut, and airways, with the highest density found in the intestine. The gut microbiota strongly influences our metabolic, endocrine, and immune systems, as well as both the peripheral and central nervous systems. Recently, a dialogue between the gut and lung microbiota has been discovered, suggesting that changes in one compartment could impact the other compartment, whether in relation to microbial composition or function. Further, this bidirectional axis is evidenced in an, either beneficial or malignant, altered immune response in one compartment following changes in the other compartment. Stimulation of the immune system arises from the microbial cells themselves, but also from their metabolites. It can be either direct or mediated by stimulated immune cells in one site impacting the other site. Additionally, this interaction may lead to immunological boost, assisting the innate immune system in its anti-tumour response. Thus, this review offers an insight into the composition of these sites, the gut and the lung, their role in shaping the immune system and, finally, their role in the response to lung cancer.

Key words: Intestinal microbiota, lung microbiota, dysbiosis, immunotherapy, probiotics

1. Introduction

The microbiota is a consortium of different microorganisms that includes bacteria (microbiota), fungi (mycobiota), viruses and protozoa [1] residing on the skin, and in the oral, pulmonary, urogenital and gastrointestinal (GI) cavities – with the gastrointestinal having the highest density of microbiota. Weighing approximately 1.5 kg, microbial residents in the GI tract outnumber human cells 10-fold and genome size 100-fold. Their functional importance for the host is undeniable involving functions that range from breaking down complex dietary polysaccharides [2] to competing with pathogens, and modulating the mucosal and immune system in general [3]. Gut dysbiosis is now considered to be an underlying cause for a wide range of GI diseases, and an emerging number of non-GI conditions such as obesity and cardiovascular disease, as well as a range of psychiatric disease [4,5]. Recent studies have also reported the effects that the intestinal microbiota exerts on the lungs. This has been referred to as the “gut-lung axis”, which in most cases is mediated by inflammation involving the translocation of bacteria and bacterial products across the GI tract barrier and into blood vessels [6]. However, data on this topic is scarce. Studies of the lung microbiota and its interconnection with other systems and organisms are an emerging field, which is rapidly accumulating evidence to demonstrate that the lungs are not in fact sterile, but contain distinct microbial communities [7]. Moreover, it appears that chronic lung diseases such as cancer are linked to a dysbiotic airway microbiota, and commonly occur alongside GI disorders [8,9]. Likewise, individuals with irritable bowel syndrome sometimes have impaired lung function [10]. This leads us to the conclusion that the axis between lung and gut can be considered bidirectional.

In this review we give an overview of the composition of both the gut and the lung, and describe the interaction between the immune system and microbiota using the intestinal tract as an example. In the case of the lungs we are still only able to speculate about any similarity. We also examine immune stimulation of the gut to observe the effects on lung immunity, inflammation and lung cancer, and finally, we discuss how these two sites might “cooperate” to achieve a productive immune and anti-cancer response.

2. Gut microbiota

The evolution of an individual’s microbiota begins at birth, with its composition becoming relatively stable after the age of two and remaining so throughout life. The GI tract is populated by more than 1,000 bacterial species. At the level of the phylum, the composition of the microbiota is similar in most healthy people. Over 90% of bacterial cells are *Firmicutes* and

Table 1 Most frequently detected bacteria in GI tract and respiratory system of healthy volunteers, or from healthy tissue samples. Results from different studies are presented by the taxa level in which they were originally detected, in order of decreasing abundance where possible. If the sampling, analysis method, or result was specific for a certain study, the reference was added adjacent to the corresponding information.

	Sample source Analysis method	Phylum	Order or family	Genus or species	Reference
GI tract	faeces	<i>Firmicutes</i> (79.4% of sequences), <i>Bacteroidetes</i> (16.9%), <i>Actinobacteria</i> (2.5%), <i>Proteobacteria</i> (1%), <i>Verrucomicrobia</i> (0.1%) ^b		<i>Faecalibacterium</i> , <i>Ruminococcus</i> , <i>Eubacterium</i> , <i>Dorea</i> , <i>Bacteroides</i> , <i>Alistipes</i> , <i>Bifidobacterium</i> ^a	Tap et al. [11]
	qPCR ^a , 16S sequencing ^b			<i>Bacteroides (vulgatus)</i> , <i>Roseburia (intestinalis)</i> , <i>Ruminococcus (bromii)</i> , <i>Eubacterium (rectale)</i> , <i>Coprobacillus (sp.)</i> , <i>Bifidobacterium (longum)</i> , <i>Clostridium (leptum, coccoides)</i> ^b	
Oral	saliva 16S sequencing	<i>Firmicutes</i> , <i>Proteobacteria</i> , <i>Actinobacteria</i> , <i>Fusobacteria</i> , <i>TM7</i> , <i>Spirochaetes</i>	<i>Pasteurellaceae</i> (5.8%), <i>Enterococcaceae</i> (2.6%), <i>Veillonellaceae</i> (2.0%), <i>Burkholderiales</i> (1.2%), <i>Lactobacillales</i> (1.1%)	<i>Neisseria</i> and <i>Streptococcus</i> (70% of sequences), <i>Haemophilus</i> (9.2%), <i>Leptotrichia</i> (2.1%), <i>Actinomyces</i> (1.9%), <i>Abiotrophia</i> (1.8%), <i>Atopobium</i> (1.6%), <i>Gemella</i> (1.6%), <i>Arthrobacter</i> (1.0%)	Lazarevic et al. [103]
Nose	swab 16S sequencing	<i>Actinobacteria</i> , <i>Firmicutes</i> , <i>Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Fusobacteria</i>	<i>Staphylococcaceae</i> , <i>Lachnospiraceae</i> [49]		Charlson et al. [104] Lemon et al. [49]
			<i>Propionibacteriaceae</i> , <i>Corynebacteriaceae</i> [104]		
Oropharynx	swab 16S sequencing	Dominated by <i>Firmicutes</i> , <i>Proteobacteria</i> , and <i>Bacteroidetes</i> , <i>Fusobacteria</i> , <i>Actinobacteria</i> , <i>TM7</i> , and <i>SRI</i> follow (oropharynx was richer and less variable than the nostril microbiota)	<i>Streptococcaceae</i> , <i>Lachnospiraceae</i> , unclassified group of <i>Clostridia</i> [49]		Charlson et al. [104] Lemon et al. [49]
			<i>Streptococcaceae</i> , <i>Veillonellaceae</i> , <i>Fusobacteriaceae</i> , <i>Neisseriaceae</i> [104]		
Esophagus	biopsy 16S sequencing	<i>Firmicutes</i> , <i>Bacteroidetes</i> , <i>Actinobacteria</i> , <i>Proteobacteria</i> , <i>Fusobacteria</i> , <i>TM7</i>		<i>Streptococcus (mitis, thermophiles, parasanguis)</i> , <i>Prevotella (pallens)</i> , <i>Veillonella (atypica, dispar)</i> , <i>Rothia (mucilaginosus)</i> , <i>Megasphaera (micronuciformis)</i> , <i>Granulicatella (adiacens)</i> , <i>Gemella</i> , <i>TM7</i> , <i>Actinomyces (odontolyticus)</i> , <i>Bacteroides</i> , <i>Clostridium</i> , <i>Haemophilus</i> , <i>Bulleidia (moorei)</i>	Pei et al. [105]
Lung	BAL ^a , brushing ^b	<i>Actinobacteria</i> , <i>Firmicutes</i> , and <i>Proteobacteria</i> ^a [106]	<i>Streptococcaceae</i> , <i>Veillonellaceae</i> , <i>Prevotellaceae</i> , <i>Micrococcaceae</i> , <i>Neisseriaceae</i> , <i>Porphyromonaceae</i> , <i>Lachnospiraceae</i> , <i>Actynomicetaceae</i> , <i>Fusobacteriaceae</i> ^a [104]	<i>Pseudomonas</i> , <i>Streptococcus</i> , <i>Prevotella</i> , <i>Fusobacterium</i> , <i>Haemophilus</i> , <i>Veillonella</i> , <i>Porphyromonas</i> ^a [62]	Charlson et al. [104] Erb-Downward et al. [62] Hilty et al. [7] Pragman et al. [106]
	16S sequencing		<i>Bacteroidetes</i> , <i>Firmicutes</i> , <i>Proteobacteria</i> , <i>Actinobacteria</i> , <i>Fusobacteria</i> ^b [7]	<i>Prevotella</i> , <i>Veillonella</i> , other <i>Firmicutes</i> , other <i>Bacteroidetes</i> , <i>Streptococcus</i> , <i>Haemophilus</i> , <i>Neisseria</i> , <i>Fusobacterium</i> , other <i>Actinobacteria</i> , <i>Staphylococcus</i> , other <i>Proteobacteria</i> , <i>Corynebacterium</i> ^b [7]	

Abbreviations: BAL = bronchoalveolar lavage.

Bacteroidetes, followed by *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*, together constituting 99% of the overall commensal microbiota [11]. Around 60 species have been identified as the “core” microbiota, mostly bacteria from the *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Ruminococcus*, *Faecalibacterium* genera, as well as a few others [11]. A summary overview of the most prevalent human GI microbiota is shown in Table 1.

Strongly correlating to long-term diet [12], different enterotypes can also be identified through a variation in the levels of one of the three most abundant genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) and *Ruminococcus* (enterotype 3), which is often less clearly distinguished [13].

The impact of the GI microbiota on host mucosal immunity

The microbiota is now considered key to the proper development, maturation and reactivity of the immune system [6,14]. Microorganisms serve as an inexhaustible source of microorganism-associated molecular patterns (MAMPs) as well as pathogen-associated molecular patterns (PAMPs). The two are recognizable on the host's cells through pattern-recognition receptors (PRRs), which include toll-like receptors (TLRs) and nucleotide-binding receptors (NODs) [15]. TLRs are conserved receptors of the innate immune system that recognize MAMPs and PAMPs among other molecules, evoking different immunological reactions depending on the type of the cell, ligand and the receptor itself (some of the most common pairs are shown in Figure 1A). TLRs, which are in direct contact with the intestinal lumen, are found not only in intestinal epithelial cells (IECs) but also on immune cells within the lamina propria, such as macrophages, dendritic cells (DCs), B cells, T cells and stromal cells. In IECs, TLR activation by microbial ligands results in epithelial cell proliferation and the expression of antimicrobial peptides, and secretion of immunoglobulin A (IgA) produced by plasma cells in lamina propria, into the gut lumen [16], as well as the expression of antimicrobial peptides. All of the above lead to enhanced intestinal barrier function and limit the possibility of microbial breach. Interestingly, some of the TLRs, such as TLR2 and TLR4, are inhibited by IEC's intracellular Toll-interacting protein (TOLLIP) (Figure 1B) when the signal comes from the intestinal lumen, which suggests a selective inflammatory response reserved to microbes that have breached the intestinal barrier [16]. NOD-like receptors, or nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs), are cytoplasmatic equivalents of TLRs that detect bacterial PAMPs entering the mammalian cell (Figure 1B). They are especially important in tissues where TLRs are expressed at low levels e.g. in GI tract epithelial cells where the cells are in constant contact

with the microbiota, and the expression of TLRs must be down-regulated to avoid over-stimulation [17].

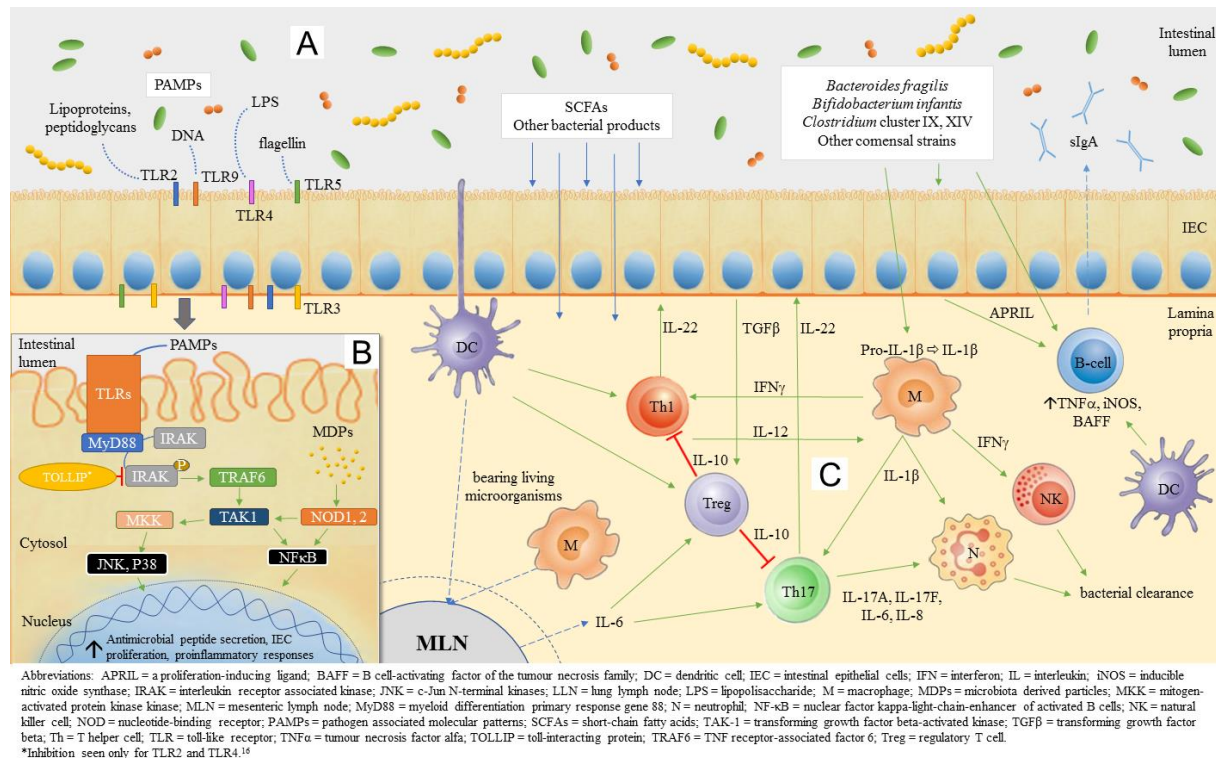


Figure 1 Interaction of the microbiota and intestinal mucosa. A) Microorganisms in the intestine provide pathogen-associated molecular patterns (PAMPs) that serve as ligands for different Toll-like receptors (TLRs) on the luminal or basolateral surface of the intestinal epithelial cells (IECs). B) TLRs stimulation activates a signalling cascade resulting in transcription factor activation and gene transcription, enhancing the cell barrier and further stimulating the immunological cells in the lamina propria. This cascade can be inhibited by toll-interacting protein (TOLLIP). *Inhibition seen only for TLR2 and TLR4 [16]. C) Commensal bacteria and their derivatives (e.g. short-chain fatty acids (SCFAs)) directly stimulate IECs (B) or can be phagocytosed by DCs and macrophages in lamina propria, carried to mesenteric lymph nodes (MLN) where they prime naïve B and T cells to mature and differentiate. B cells become plasma cells and produce IgA that is secreted into the intestinal lumen (sIgA). T cells profile into Th17 and Th1, with proinflammatory tendency, activating additional effector cells as neutrophils, resulting in bacterial clearance. There is also differentiation to Treg cells having anti-inflammatory properties and controlling inflammation.

Commensal microorganisms can enter intestinal lamina propria in several ways: through an opening in the barrier as a result of injury, or through active sampling by DCs or M cells. In any case, microorganisms in the lamina propria are either phagocytosed and eliminated by macrophages [18], or engulfed by DCs (along with B cells, both are considered the “professional” antigen presenting cells (APCs)) and are carried live to the mesenteric lymph nodes (Figure 1C). Recognition of infected apoptotic cells and bacteria results in the upregulation of interleukin 6 (IL-6), which drives the differentiation of proinflammatory T-helper-IL-17-producing (Th17) cells. Th17 cells primarily produce two main members of the

IL-17 family, IL-17A and IL-17F which are involved in the recruitment, activation, and migration of neutrophils [19], with granulocytes playing an important role in bacterial clearance. Commensal-bearing DCs also induce the production of protective secretory immunoglobulin A (sIgA) in activated B cells (Figure 1C) [20], i.e. plasma cells. This sIgA is then distributed across all mucosal surfaces through the recirculation of activated B and T cells. Commensal bacteria also directly promote the expression of factors involved in the induction of IgA⁺ B cells (Figure 1C) [21], their survival and, interestingly, production of the more resistant form of sIgA, exerting its stability and antimicrobial properties [22]. Through this constant “priming” across several layers, microbiota maintains the immune system’s readiness and reactivity, making it more capable of a quick and effective response when needed.

Specific populations of commensal bacteria e.g. *Bacteroides fragilis*, *Bifidobacterium infantis*, *Clostridium* cluster IV and XIVa, also induce so-called regulatory T cells (Tregs), a subset of forkhead box P3 positive (FoxP3⁺) CD4⁺ T-helper lymphocytes that maintain gut homeostasis by stimulating the production of anti-inflammatory cytokine IL-10 [23]. These cells serve as a kind of counterbalance to Th17 response, controlling the scope of its reaction and proinflammatory cytokine production. Thus, depletion of Tregs leads to the abnormal expansion of CD4⁺ Th cells, resulting in robust IL-17 and interferon gamma (IFN γ) responses in the colonic lamina propria, to produce intestinal inflammation.

To understand more precisely which commensal microbiota has this immunostimulatory effect, the well-known *Bifidobacterium* probiotic group was tested for its ability to induce the full maturation of human peripheral blood mononuclear cells (PBMCs) into DCs. Twelve *Bifidobacterium* strains descending from 4 probiotic species (*Bifidobacterium longum*, *B. breve*, *B. bifidum*, and *B. animalis* subsp. *lactis*) were tested, and each was induced to full maturation into DCs but using different Th preferences (Th1 or Th17), depending on the type of *Bifidobacterium* strain with which they were incubated. Also, cell-free culture supernatants were poor inducers of maturation [24], leading us to conclude that live bacterial cells are necessary to induce efficient maturation and antigen presentation in DCs with this type of bacteria.

Likewise, introducing probiotic strains such as *Bifidobacterium lactis* into healthy elderly volunteers with fully developed immune systems, resulted in a significant increase in the proportion of mononuclear leukocytes, the phagocytic capacity of mononuclear and polymorphonuclear phagocytes, and the tumoricidal activity of NK cells [25], highlighting the

importance of this specific immune-boosting species whose presence directly impacts immune status.

Immunity and inflammation are not necessarily affected by bacterial cells, but may be influenced by bacterial products. Bacterial products that have a significant effect on overall host status surely included short chain fatty acids (SCFAs), by-products of the microbial fermentation of dietary fibre. Amongst others, *Bacteroides*, *Bifidobacterium*, *Propionibacterium*, *Eubacterium*, *Lactobacillus*, *Clostridium*, *Roseburia*, *Prevotella* are all remarkable SCFA producers [26]. Fermentation and SCFA production are thought to inhibit the growth of pathogenic organisms by reducing luminal pH [27]. The most abundant SCFAs are acetate, propionate (found mostly in the small and large intestines) and butyrate (found mostly in the caecum and colon), which are primarily derived from carbohydrates [28]. SCFAs have their specific receptors both on leukocytes and on endothelial cells. Known as two formerly orphan G protein-coupled receptors, GPR41 is found in a wide range of tissues including in neutrophils, while GPR43 is shown to be highly expressed in immune cells [29]. GPR109a, the third receptor found in the colon epithelium and immune cells, is butyrate-specific, and closely associated with the anti-inflammatory effect [30]. Butyrate is one of the most important SCFAs with members of the *Firmicutes* phylum as major butyrate producers, harbouring genes for the acetyl-CoA pathways. Other than as the main energy source for the intestinal epithelium and its role in barrier integrity [31,32] following selective transport into the colon epithelium, butyrate manifests broad anti-inflammatory activities such as immune cell activation, proliferation, migration, adhesion, cytokine expression, and cancer cell apoptosis [14,30]. These are mostly attributed to its function as a histone deacetylase (HDAC) inhibitor. HDAC inhibition influences the acetylation not only of histones (FoxP3 locus in Tregs, important for their maturation) [33], but also of major transcription factors such as nuclear factor kappa-light-chain-enhancer for activated B cells (NF- κ B), or signal transducer and activator of transcription 3 (Stat3) [34,35], major proinflammatory pathway factors affecting the proinflammatory cytokine secretion profile in immune cells [36] and decreasing the proliferation and apoptosis of tumour cells [37].

As mentioned previously, changes to the gut microbiota are related to, for example, changes in diet, antibiotic administration, chemotherapy, and a person's general immune status. Whether with a transient or permanent effect, these changes often lead to dysbiosis, with an altered ratio of beneficial bacterial species (e.g. *Lactobacillus* sp., *Faecalibacterium prausnitzii*, etc.) and/or an overgrowth or population shift of other species [14]. In this event, expanded indigenous

microorganisms (now potential opportunistic pathogens), may also produce DNA-damaging superoxide radicals and genotoxins in significant concentrations and induce innate immune mediated proinflammatory pathways [38], directly damaging cells, and promoting malignant transformation by inducing chromosomal and microsatellite instability, CpG island methylation, epigenetic alterations, and post-translational modifications, which weaken the immune response and increase the risk of cancer [39].

Apart from the direct pathological effect, an absence of the appropriate microbial composition in the immune system's early development has more far-reaching effects. This is evident from studies on mice reared in germ-free (GF) conditions. These animals have impaired GI-driven immune development, characterized by smaller Peyer's patches, fewer CD8 $\alpha\beta$ intraepithelial lymphocytes, underdeveloped isolated lymphoid follicles, a lack of primed T cells, lower levels and impaired production of mucosal IgA antibodies, and active IL-10-mediated inflammatory hyporesponsiveness [40,41]. Also, mice with colitis-associated cancer (CAC) that lacked microbiota were unable to process pro-IL-1 β and pro-IL-18 (interleukins in this case necessary for a desirable inflammatory reaction) into their mature forms, resulting in increased tumour burden [42]. That said, we can see that the composition of "healthy" microbiota is crucial to the proper development of the immune system's basic structures. Unless fully developed, their ability to exchange information and their reactions to the "outside" world are compromised. This state could be characterised as similar to anergy: the signal is present but the immune system does not respond, as exemplified in the case of CAC mice.

3. Lung microbiota

The human respiratory tract is the primary and continuous entry portal for numerous microorganisms and particles, such as viruses, bacteria, or fungi. These are primarily airborne, but can also be transferred through saliva. Below the vocal cords, the human airways harbour bacteria and other microbes in rich surroundings [43] that are distinct in composition from the microbiota seen at other sites (in the nasal and oral cavity, gut, skin, and vagina). Despite being less populated compared to the GI tract, the lung microbiota includes a range of microorganisms, mostly seen through the use of bronchoalveolar lavage (BAL) fluid, or tissue samples. Since lung microbiota exploration has rather young and non-uniform protocols, it is crucial to bear in mind that the type of sample (lavage, tissue), sampling method, and possibility of cross-contamination during sampling between distinct parts of the airway, influence final results [44,45]. Therefore, due to the paucity of overall studies in this field, one must be careful

to take detailed methodology and its possible advantages and disadvantages into account. Studies that analyse lung tissue acquired through sterile surgical explant have also been carried out, and they all report that the lower respiratory tract contains a microbiome that is distinct from, but related to, that of the upper airways [46].

In 2014 Dickson et al. [47] proposed an adapted island model for lung biogeography, suggesting that more distal lung bacterial communities are less rich and more dissimilar to their upper respiratory tract source community. Here, microbial composition is determined by the rate of microbial immigration into the airways, their rate of elimination (e.g. by coughing or immune defences), and the rate at which different community members are reproduced [48]. Temperature, oxygen tension, pH, nutrient density, local anatomy, and host defence are spatially heterogeneous across the airways and the lungs, all of which affect local microbiological growth conditions.

Starting with the upper respiratory airways, the nostril is dominated by *Firmicutes* and *Actinobacteria*; *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* are prevalent in the oropharynx [49]. In the lung the most common phyla consistently observed are *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. The nasal microbiota seems to more closely resemble that of the skin than that of the lungs and contributes little to lung communities [50]. The coming years are likely to bring the development of new methods able to minimize cross-contamination (as seen for endoscopy [51]), which is the biggest problem in lower respiratory tract sampling that, along with metagenomic analysis (of both cultivable and non-cultivable bacteria), will yield more precise results in terms of the bacterial population found at certain airway depths. An overview of different studies investigating the composition of the respiratory system microbiota is presented in Table 1.

The ecological determinants of the lung microbiota (immigration, elimination, and regional growth conditions) change during acute and chronic lung disease, as seen in chronic obstructive pulmonary disease (COPD) (often a precancerous inflammatory state) and lung cancer (Table 2) [7,44]. Whether the observed dysbiosis is a cause, consequence or simply a co-evolving factor, still needs to be elucidated, but its likely role will be individually connected to the pathology and its aetiology. However, it is known that smouldering inflammation (caused by lung injury, pathogen colonization, or intrinsic factors) is often a common starting point that leads to subsequent cancer development [52].

The microbial factors that may be responsible for lung cancer development are still not well known, unlike the many genetic predispositions and mutations that underlie the different types of lung cancer [53]. This is why, for now, it is possible to correlate the non-genetic development of lung cancer with the well characterized development of COPD, a chronic inflammatory state where initial lung injury, whatever its cause, creates an opening for microbial dysbiosis and colonization, thus worsening the overall condition and often leading to the cancer state.

Inflammation of the lung is associated with a loss of epithelial integrity and results in the “leakage” of serum proteins into the airways [54]. Formyl peptides, cleavage products of bacterial or mitochondrial proteins, as well as other bacterial products, serve as powerful chemoattractants for both the neutrophils and monocytes that emigrate from alveoli [55]. Although essential in pathogen clearance and having a tumoricidal effect [56], neutrophil influx and degranulation in the airways and lung parenchyma contribute to chronic inflammation, parenchymal lung damage, and progressive small airway obstruction, due to the loss of alveolar attachments and lung elasticity [57], as explained by Sethi’s vicious circle hypothesis [58]. *In vitro*, their enzymes, serine proteinases (elastase, cathepsin G and proteinase 3), and defensins markedly affect the integrity of the epithelial layer, decreasing the frequency of the ciliary beat, increasing mucus secretion, and inducing the synthesis of epithelium-derived mediators such as neutrophil chemoattractant chemokine IL-8 from respiratory epithelial cells [59].

Obstruction of the lumina with mucus introduces pockets of increased temperature and decreased oxygen tension, selectively favouring the growth of well-known disease-associated microbes [60,61]. This dysbiotic shift can be characterized by a move away from the *Bacteroidetes* phylum, often to *Proteobacteria* (e.g. *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Moraxella catarrhalis*) [62,63], and sometimes to *Firmicutes* (e.g. *Streptococcus pneumoniae*, *Staphylococcus aureus*) [44]. The same growth effect was observed with a generation of intra-alveolar catecholamines and inflammatory cytokines [64]. If airway colonization becomes persistent, it further promotes chronic inflammatory response, affecting the elastase-anti-elastase balance in the lung, which was shown to vary 80-fold with changes to the airway bacterial load [65]. A higher bacterial load consequently induces higher overall IL-8 and other blood circulating inflammatory cytokine levels, greater inflammation, oxidative stress and greater forced expiration volume (FEV) decline [66].

Lung microbiota was also shown to vary according to clinical endpoints. In non-malignant lung tissue from advanced stage cancer, alpha diversity had increased, whilst it had decreased in the tumour lung tissue. Also, the interaction between the upper and lower airways involving the

Table 2 Most frequently detected bacteria in the lung of patients suffering from their respective diseases. Results from different studies were presented by the taxa level in which they were originally detected, in order of decreasing abundance where possible. If the sampling, analysis method, or result was specific for a certain study, the reference was added adjacent to the corresponding information.

Disease	Sample source Analysis method	Phylum	Order or family	Genus or species	Reference
Lung COPD	BAL ^a , sputum ^b , lung tissue ^c	<i>Proteobacteria</i> (44%) and <i>Firmicutes</i> (16%) followed by <i>Actinobacteria</i> (13%), with <i>Bacteroidetes</i> , <i>Fusobacteria</i> , <i>Tenericutes</i> , <i>SRI incertae</i> , <i>TM7</i> , and <i>Synergistetes</i> identified in lower proportions (<3%) ^{b,d} [107]		<i>Haemophilus influenzae</i> , <i>Pseudomonas aeruginosa</i> , <i>Moraxella catarrhalis</i> , <i>Streptococcus pneumoniae</i> ^{b,d} [107] Significant increases of <i>Streptococcus pneumoniae</i> , <i>Klebsiella pneumoniae</i> and <i>Pseudomonas aeruginosa</i> ^{b,e} [63]	Erb-Downward et al. [62] Garcia-Nuñez et al. [107] Hilty et al. [7] Sze et al. [44] Wu et al. [63]
		Increase in <i>Proteobacteria</i> (sometimes <i>Firmicutes</i>), decrease in <i>Bacteroidetes</i> and <i>Firmicutes</i> ^{c,d} [44]		<i>Streptococcus</i> , <i>Abiotrophia</i> , <i>Rothia</i> , <i>Tropheryma</i> , <i>Actinomyces</i> , <i>Peptostreptococcus</i> , <i>Serratia</i> , <i>Capnocytophaga</i> , <i>Leptotrichia</i> , <i>Kingella</i> , <i>Dysgonomonas</i> ^{a,d} [106]	Pragman et al. [106]
	16S sequencing ^d , qPCR/DGGE ^e , bacterial culture ^f	In moderate COPD mostly <i>Actinobacteria</i> and <i>Proteobacteria</i> In severe COPD mostly <i>Actinobacteria</i> and <i>Firmicutes</i> ^{a,d} [106] <i>Proteobacteria</i> , <i>Bacteroidetes</i> , <i>Firmicutes</i> , <i>Actinobacteria</i> , <i>Fusobacteria</i> ^{b,d} [7]		Significant abundance of <i>Pseudomonas</i> and <i>Haemophilus</i> ^{a,d} [62] <i>Prevotella</i> , <i>Haemophilus</i> , <i>Streptococcus</i> , <i>Neisseria</i> , other <i>Proteobacteria</i> , <i>Veillonella</i> , other <i>Firmicutes</i> , other <i>Bacteroidetes</i> , <i>Fusobacterium</i> , other <i>Actinobacteria</i> , <i>Staphylococcus</i> ^{b,d} [7]	Wu et al. [63] Pragman et al. [106]
Lung cancer	Sputum ^a , saliva ^b anaerobic culture ^c , 16S sequencing ^d		<i>Flavobacteriales</i> , <i>Burkholderiales</i> , <i>Campylobacterales</i> , <i>Spirochaetales</i> (more abundant) and <i>Bacteroidales</i> (less abundant) ^{b,d} [67] <i>Veillonellaceae</i> significantly overexpressed whereas <i>Lachnospiraceae</i> underexpressed ^{b,d} [67]	<i>Actinomyces</i> spp., <i>Peptostreptococcus</i> spp., <i>Eubacterium lentum</i> , <i>Veillonella parvula</i> , <i>Prevotella</i> spp., <i>Bacteroides</i> spp., <i>Lactobacillus jensenii</i> ^{a,c} [108] <i>Capnocytophaga</i> , <i>Selenomonas</i> , and <i>Veillonella</i> were found to be more abundant in both SCC and AC ^{b,d} [67]	Rybojad et al. [108] Yan et al. [67]

Abbreviations: COPD = chronic obstructive pulmonary disease; BAL = bronchoalveolar lavage; DGGE = PCR-denaturing gradient gel electrophoresis; SCC = small cell carcinoma; AC = adenocarcinoma.

microbial population present in lung cancer is clearly shown in the study carried out by Yan et al. [67]. The study showed that there is a high degree of specificity in patients with either small cell carcinoma (SCC) or adenocarcinoma (AC), compared to controls based on bacteria isolated from saliva (Table 2).

As mentioned above, IL-6 and IL-8 are cytokines that become elevated during inflammatory stress. They are involved in tumorigenesis by acting directly on lung epithelial cells to stimulate the NF- κ B-1 pathway [68]. Additionally, IL-6 and IL-8 are expressed by premalignant or senescent lung cancer cells [69]. They may act in an autocrine and/or paracrine fashion to stimulate cancer cell proliferation [70], migration, and invasion [71]. In bronchoalveolar carcinoma, tumour cells were a main source of IL-8 and the presence of an increased number of neutrophils in BAL fluid was correlated with the IL-8 level in BAL, and associated with a poor outcome [72]. In a case study carried out by Pine et al. [73], increased levels of both serum IL-6 and IL-8 were associated with lung cancer, but only the IL-8 level was associated with lung cancer risk several years prior to diagnosis.

To summarize, the appearance of dysbiosis or malignancy is likely the product of a dynamic interaction between various immune, microbial, and environmental factors. At least one of these acts as an initiator but others often readily follow. This is why it remains difficult to reach any conclusions regarding the true aetiology of disease and what might be the best intervention and, more importantly, prevention approach.

4. A bidirectional concept of the gut-lung axis

Recently, we have reached a greater understanding of microbial influence on the complex and interconnected axis between gut and lung. This stems from the simple fact that ingested microorganisms can access both sites – from gastrointestinal tract microbiota that enter the lung through aspiration [74] to the more “internal” influence, which shows improved lung function and pathogen clearance following the transplantation of faecal microbiota [6].

This interaction can be mediated in different ways – by the microbiota and its products, or via immune cells (Figure 2). According to the “gut-lymph” theory of Samuelson et al. [14], there are sufficient macrophages and other immune cells in the intestinal submucosa or the mesenteric lymph nodes that contain a majority of translocating bacteria. Surviving bacteria, cell wall fragments, or the protein parts of dead bacteria escaping with the cytokines and chemokines produced in the gut, travel along the mesenteric lymphatic system to the cisterna

chyl, and subsequently enter the systemic circulation. Access to pulmonary circulation may lead to DC and macrophage activation as well as the priming of T cells and their differentiation.

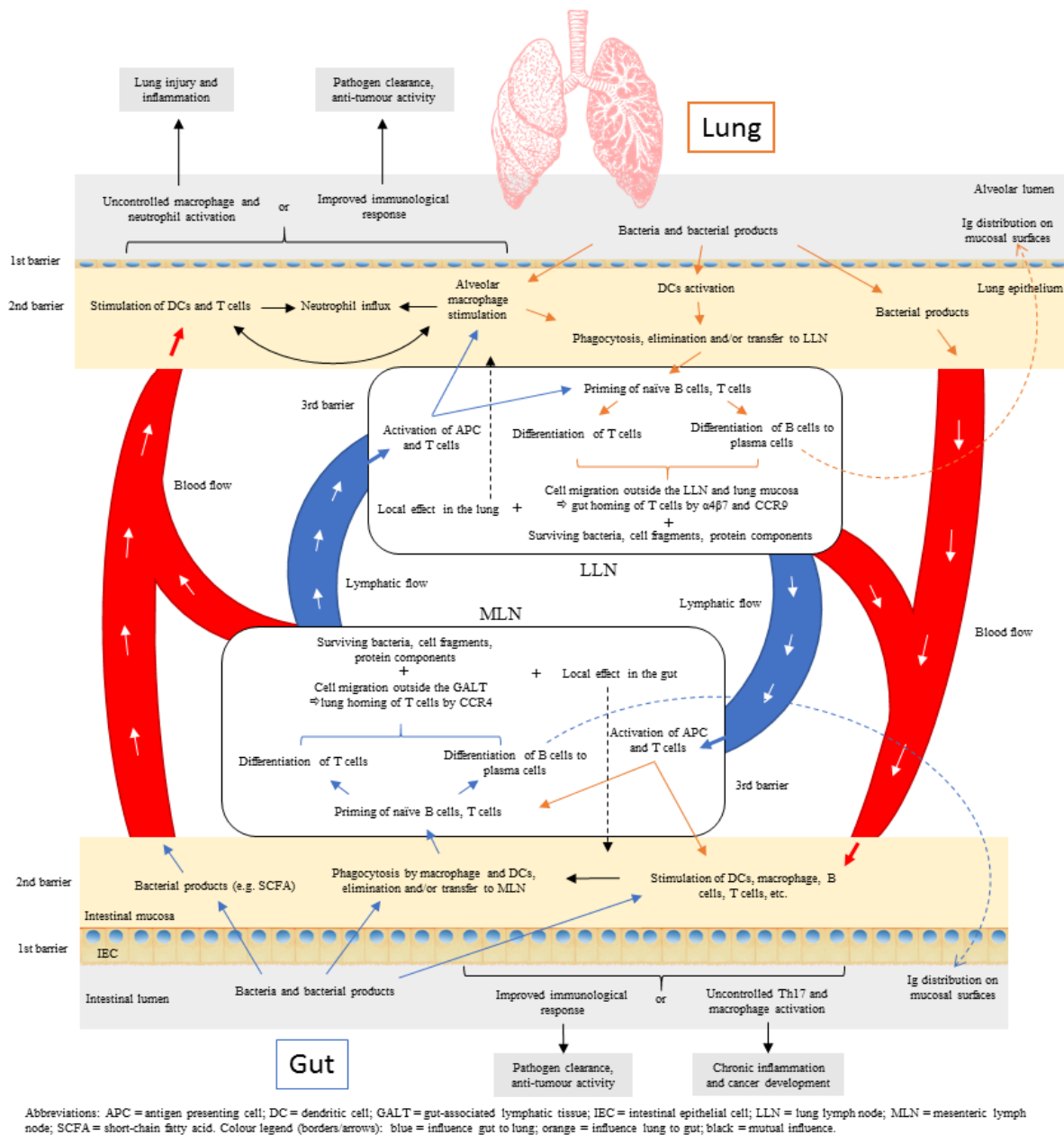


Figure 2 Proposed pathways of the gut-lung interaction. Microbiota and its products that enter intestinal mucosa (blue arrows) are phagocytosed and transferred to mesenteric lymph nodes (MLN) by antigen presenting cells (APC), where they stimulate priming of the T and B cells. Once activated, with the expression of proper homing receptors, these cells can migrate back to the original site (intestinal mucosa) (black dashed arrow), or to distal locations such as the lung epithelium and lung nodes through lymphatic and blood circulation. There, they can directly act on their target or continue to stimulate other immune cells. On the other hand, bacterial products from the intestinal mucosa or surviving bacteria can also reach the lung by blood or lymphatics to stimulate the immune system in the same way as they would have done in the intestinal tract. Depending on the tissue pre-stimulation, type of stimulus, and local and general immunological status, the result can be either positive, as effective bacterial clearance or anti-tumour activity, or over-inflammatory response, promoting further tissue damage,

pathogen colonization, and tumour progression. The same schema is proposed in the other sense, beginning with the lung mucosa and finishing with distal effect on the gut. Although not known for the moment, there is also a possibility that bacterial products of the lung microbiota can exert their effect in the intestinal mucosa, being delivered in the same way as explained above.

Another way to influence the pulmonary region is through the migration of immunological cells. As previously mentioned, translocated microorganisms and their parts within the lamina propria are transferred to the mesenteric lymph nodes by antigen presenting cells (APCs), and used for priming naive B and T cells. Activated B cells capable of producing antigen-specific immunoglobulins, i.e. plasma cells, will not only produce immunoglobulins *in situ*, but will reach draining lymph nodes and other mucosal tissues, thereby spreading immunological “information”. The constitutive entry of antigen at steady-state stimulates inflammasome conversion of pro-IL-1 β and pro-IL-18 into active form, in other words it switches off our innate ability to produce IL-10 and other anti-inflammatory molecules leading to DC migration to local lymph nodes, and the priming and differentiation of T cells. The latter can subsequently migrate out of the gut-associated lymphatic tissue (GALT) and reach both mucosal and peripheral non-mucosal tissues, including the bronchial epithelium, thus modifying the immunological response which is dependent on the induced cell profile (to Th1, Th2, etc.) [75,76], and improving the immunological response against pulmonary pathogens [77].

Although this theory explains the unilateral interaction, it is reasonable to speculate that this axis works in precisely the same way when it originates in the lung mucosa and lung lymph nodes (Figure 2). Further, lung DCs *in vitro* have the option to imprint the expression of gut-homing integrin $\alpha 4\beta 7$ and CCR9 (lung-homing integrin is CCR4 [78]) on co-cultured T cells *in vitro*, and on adoptively transferred cells *in vivo*, which guides their migration to the GI tract [79].

4.1. Influence of the gut microbiota on the lung

The composition of “healthy”, or rather balanced, gut microbiota is shown to have a serious influence on the effectiveness of lung immunity. GF mice, devoid of their intestinal microbiota during the development of their immune system, show impaired pathogen clearance in the lung, which results in their growth and dissemination [41]. At this stage, it is also important to consider that the lungs of these mice are also germ-free [80], devoid of all microbiota that might normally play a role in stimulating lung immunity. Modified alveolar architecture also results, and thus both factors modify the response to pathogen infection. The same observations were made as with the infection of GF animals, when wild phenotypes were treated with antibiotics, thus disturbing the intestinal homeostasis, after they had been challenged with bacterial or viral

microorganisms [81,82]. If animals had been boosted with lipopolysaccharide (LPS) following antibiotic treatment, they were better able to cope with the lung infection and consequently had reduced mortality. Population studies followed these findings with respect to the importance of preserving gut microbiota, showing that increased use of penicillins, cephalosporins, macrolides and quinolones correlated with an increased risk of lung cancer in humans [83]. Here we can speculate that various antibiotic treatments eradicate the bacterial populations required for effectively priming T lymphocytes with anti-tumour properties, while at the same time making room for other opportunistic pathogens to colonize both the gut and the lung.

Interestingly, modified gut microbiota not necessarily characterized as dysbiotic may also influence immune response efficiency, as seen in obese mice. These mice had an impaired expression of cytokines in their lungs (IFN α , IFN β , IL-6, TNF α) and significantly decreased mRNA of IFN γ , interleukin 2 receptor subunit beta (IL-2RB) and perforin 1 (Prf1). All the criteria were improved following daily supplementation with a probiotic strain of *Lactobacillus gasseri* [84].

Nutrition may also impact microbial development and the composition of our respiratory tract microbiota [85]. A high-fibre diet in mice has been shown to increase SCFA circulating blood levels (but no traces in the lung itself), and has been shown to be responsible for increased protection against allergic inflammation in the lung (reduced inflammatory cell infiltration), followed by a change in the intestinal and, to a lesser extent, the airway microbiota [86].

The above-mentioned findings clearly show how important the overall composition of the intestinal microbiota is for a productive immunological response in the lung. Lack of an appropriate stimulus during the developmental phase, as during infection, will disable a quick and effective immune reaction, resulting in pathogenic colonisation, increased susceptibility to infection, damage, the possible development of cancer, and increased mortality. At the same time, just one single strain, bacterial part or product can turn the tables and provide the boost needed to stimulate the correct immune response.

4.2. Influence of the lung microbiota on the gut

Unlike the local and systemic influence of intestinal microbiota, the influence of lung microbiota, its products, and their circulation are yet to be properly assessed. One study reported that non-absorbable tracer deposited into the nasal cavity of mice can be found in the GI tract a short time later [87]. Also, Sze et al. [43] showed that, in mice, even acute exposure to a single dose of intratracheal LPS disrupts the airway microbiota, leading to translocation of these

bacteria into the bloodstream. Within 24 hours the caecal microbiota is also disturbed, leading to a sharp increase in the total bacterial load. It still remains unclear as to whether this effect is due to the direct interaction of translocated pulmonary and residential intestinal microbiota via immune cell or cytokine mediation, or to microbial products that reach the gut.

4.3. Microbiota and cancer via the immune system

Due to a number of genetic alterations resulting in the loss of normal cellular regulatory processes, cancer cells express neoantigens that are tumour-specific and distinguish tumour cells from healthy cells. The importance of the gut microbiota in anti-cancer response has been described by Chen et al. [88] through the concept of a cancer-immunity cycle. The cancer-immunity cycle starts with the capture of neoantigens from cancer cells by DCs. For an anti-cancer response to take place, this must be accompanied by another signal, such as proinflammatory cytokines, factors released by dying cancer cells or by gut microbiota components. The goal here is to reduce peripheral tolerance to tumour antigens. Following processing, DCs present captured neoantigens to T cells, thus resulting in their priming and activation to create effector T cells against cancer-specific antigens. At this point, the balance between T effector and T regulatory cells is crucial in determining the nature of the immune response. The now activated T effector cells travel to the tumour site, invade the tumour bed and, by recognizing specific tumour antigens, bind and kill cancer cells. Problems arise when tumour antigens are not detected, meaning that DCs and T cells treat antigens as ‘self’ rather than foreign. In this case, a Treg response rather than an effector response results. Homing of T cells to tumour may not be correct either. T cells may be inhibited from infiltrating the tumour, or (more importantly) factors in the tumour microenvironment may suppress any effector cells that are produced. There are two main negative regulators of T cell responses: checkpoints in lymphoid organs (CTLA-4), and immunostats within the tumour beds (PD-L1:PD-1). Programmed cell death ligand 1 (PD-L1) is a molecule expressed on tumour cells or on tumour-infiltrating immune cells, which binds programmed cell death protein 1 (PD-1) expressed on effector CD8⁺ T lymphocytes, blocking the secretion or production of the cytotoxic mediators needed to kill tumour cells within the tumour beds. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), expressed on Tregs, acts as the major negative regulator of the priming and activation of effector CD8⁺ T cells inside the lymphoid organs, by binding its CD80 and CD86 ligands on APCs. The presence of these suppressive factors explains the limited activity of previous immune-based therapies. The goal of current cancer immunotherapy, using anti-PD-L1:PD-1 and anti-CTLA-4 antibodies, is to initiate or reinstate a self-sustaining cycle of

cancer immunity, enabling it to amplify and propagate without creating an unrestrained response.

To create a higher response to neoantigens, the immune system's peripheral tolerance must be reduced. It is already known that the commensal microbiota induces the generation of CD4⁺ T cells against their own antigens [89] thereby limiting the systemic dissemination of commensal bacteria [90]. The same antigen cross-reactivity effect, or super antigen-driven response, accounts for T cell-dependent tumour regression. As suggested by Viaud et al. [36], and based on recent studies by Iida et al. [91] and Viaud et al. [92] in mice, Th17 cells and memory Th1 cells elicited against commensal bacteria might preferentially accumulate in an inflammatory tumour microenvironment, already primed by bacterial products or ligands for PRRs. Based on these studies, Zitvogel et al. [93] explain the long-range effect of microbiota through two signal hypotheses. Signal 1 hypothesis suggests a phenomenon of antigen mimicry or cross-reactivity. That is, certain microbial antigens from the bacterial species that pass the intestinal barrier and are used for T cell priming could closely resemble tumour antigens, thus promoting better immune system reactivity and anti-tumour response, i.e. immunosurveillance. In signal 2 hypothesis, by interacting with PRRs after passing the intestinal barrier, microbiota can stimulate the production of a diverse palette of cytokines and interferons, and determine whether it will elicit a proinflammatory, immunostimulatory or immunosuppressive response. Also, there is some evidence that commensal-specific Tregs are capable of switching to effector inflammatory Th17 cells after sensing the disruption of the mucosal barrier. Because microbial products, metabolites, effector cells, and cytokines are able to travel, this stimulation is not necessarily confined to just the gut.

Observing these effects, it is interesting to speculate that at least a transient disruption of intestinal barrier functions and microbiota translocation is a primary factor in shaping the relationship between the gut microbiome, the immune system, and cancer.

4.4. Probiotics and the lung

Probiotics, best known in nutritional therapy, are defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host” [94]. In the intestine they mainly refer to the genera *Lactobacillus* and *Bifidobacterium*, and include many different strains such as *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. johnsonii*, *L. fermentum*, *L. reuteri*, *L. plantarum*, *B. longum*, *B. breve*, *B. bifidum*, *B. animalis* subsp. *lactis*, etc. The same genus does not necessarily involve the same characteristics, due to the great genomic differences between species and also within the different strains of the same species [95].

Although the first evidence of probiotic influence on lung cancer was seen in 1985 [96], probiotics only recently re-emerged in the field of lung oncology as a possible new therapy, and is already showing highly promising results. Using different mouse lung cancer models, conventional therapies (a combination of platinum-based agents with paclitaxel, gemcitabine, vinorelbine, or docetaxel, which all have high toxicity [97]) were combined with specific probiotic strains or, conversely, the antibiotic eradication of microbiota, to assess the effect of chemotherapy.

In a lung adenocarcinoma viral model, when vancomycin was used to eradicate gram-positive bacteria it compromised the efficacy of cyclophosphamide (CTX)-based chemotherapy, and correlated with a reduced intratumoural CD8⁺ T effector/ FoxP3⁺ regulatory T cell ratio [98]. Likewise, in mice treated with cisplatin combined with an antibiotic cocktail of vancomycin, ampicillin, and neomycin, tumour size was larger than that found in mice receiving a single treatment of cisplatin [99]. Taking these examples, we can readily conclude that the presence of conventional intact microbiota is crucial for effective chemotherapy.

On the other hand, feeding mice orally with *L. acidophilus* (Lewis lung cancer model) treated with cisplatin decreases the size of tumours and improves the survival rate. Enhanced anti-tumour response is also achieved through upregulation of IFN γ , granzyme B (GzmB), and Prf1 expression [99] following probiotic supplementation.

Recently, there was considerable interest in evaluating the role of gut microbiota in lung cancer therapy using immune checkpoint inhibitors. One of the first studies of this principle was done using a mouse melanoma model, but is readily applicable to other cancer types, as shown in the study. Here, oral administration of a *Bifidobacterium* cocktail (*B. bifidum*, *B. longum*, *B. lactis* and *B. breve*) on its own improved tumour control to the same degree as PD-L1-specific antibody therapy (checkpoint blockade) [100]. When the two treatments were combined it virtually abolished tumour outgrowth. Improvement was seen in immune responses upstream of T cells, at the level of host DCs. Their augmented function enhanced CD8⁺ T cell priming and accumulation in the tumour microenvironment. The percentage of MHC II^{hi} DCs was also increased. With *Bifidobacterium* treatment, 760 genes were upregulated, including cytokine-cytokine receptor interaction, CD8⁺ T cell activation and costimulation, DC maturation, antigen processing and cross presentation, the chemokine-mediated recruitment of immune cells to the tumour microenvironment, and type I interferon signalling.

Vétizou et al. [101] reported that the oral feeding of GF mice with *Bacteroides fragilis* induced Th1 immune responses in tumour-draining lymph nodes and promoted the maturation of intratumoural DCs. This was, as the authors suggest, due to the cross-reactivity of the bacterial and tumour epitopes, which led to the restoration of the therapeutic response of GF tumour bearers to CTLA-4 antibody treatment. Antibody therapies such as this prevent inactivation of the CD8⁺ T cells by binding to the CTLA-4. Daillère et al. [102] noted that *Enterococcus hirae* and *Barnesiella intestinhominis* specific-memory Th1 cell immune responses selectively predicted longer progression-free survival in advanced lung cancer patients treated with chemo-immunotherapy. Both strains represent valuable "oncomicrobiotics" improving the efficacy of the most common alkylating immunomodulatory compound.

To summarize, as scientists delve deeper, the beneficial effects of probiotics on the immune system continue to emerge. As seen, certain strains have the power and ability to stimulate anti-tumour response or to simply stimulate the immune system to show lower tolerance, thus promoting higher reactivity and tumour eradication. The future objective is to find the optimum probiotic cocktail that may one day completely substitute conventional therapies, thereby obtaining equal or better success, and lowering toxicity, one of the biggest problems in cancer treatment.

5. Conclusion

The importance of the gut microbiota and its composition has long been recognized, for digestion as well as for overall wellbeing. Recently, the presence of the lung microbiota and the role it plays, in health and in disease, has been receiving attention. The lung and gut microbiota, both continually reseeded through interaction with the environment, modulate our local and systemic immunity. More than simply two distinct microbiota, they are now seen as functioning in dialogue, altering previous ideas of airway sterility and the existence of a "barrier" between the two compartments, due to their perceived distance or functional differences. By providing stimulating signals through its epitopes or products (such as SCFA butyrate), the gut microbiota directly enhances the intestinal barrier. Likewise, it stimulates the priming and maturation of T and B cells, ensuring improved microbial clearance and mucosal protection through antibodies. This effect is not only retained in the intestinal system but is spread along other mucosal surfaces by means of lymphatic and blood circulation, influencing distal site immune response. So, even though the antigen was introduced in the gut, an immunological response can also be elicited in the lung, although there was no direct prior contact with the antigen, and vice versa.

Bacteria and their products that go through the first immunological barrier also reach distal sites through the lymphatic system and blood, and modulate the immune response at the remote site. The site where the first encounter between the immune system and microbial antigens took place is also important, since it influences reactivity and the influx of these cells into other tissues. Applying prebiotics to target a specific microbial group could be a good way to restore “healthy” microbial composition, which will consequently increase intestinal barrier function and stimulate the immune system. The relevance of natural microbial support in chemotherapy effectiveness or replacement has already been demonstrated. In future, further discoveries will surely be made in this new and exciting area of research, adding to the complexity of, but also clarifying the reasons behind, this axis; opening ideas to new or enhanced therapies based on the natural behaviour of the organism; increasing longevity; and decreasing therapeutic side-effects or the effects of disease itself.

6. References

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Thesis objectives and hypotheses

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Several studies on microbiota and cancer models in animals (Daillère et al. 2016; Sivan et al. 2015; Vétizou et al. 2015; Viaud et al. 2013) introduced a new aspect in cancer treatment, emphasizing the importance of the balanced gut microbiota and presence of specific genera to stimulate anti-cancer response in different cancers. However, translational application is still not met, since the mentioned studies in animal models are quite recent. Additional inconvenience is that in humans apart from the best-described gut microbiota, the connexion between microbiota and immune response is still in its early research phase. Moreover, interaction with cancer is at the moment based on direct cause-consequence relations as seen in *Fusobacterium nucleatum* (Rubinstein et al. 2013; Yu et al. 2017), while other distant effects of bacteria or their products on cancer are not known. Despite being the leading cause of death by cancer (International Agency for Research on Cancer (IARC) 2014; International Agency for Research on Cancer 2019), lung cancer belongs to one of the poorly characterised cancers in terms of local microbiota and consequentially, in interaction of local microbiota and tumour immune response. Interestingly however, the link between distant microbiota as of the gut with the local lung immune response was already evoked in the form of the “gut-lung” axis theory, suggesting that gut microbiota could be crucial in host’s response to lung cancer. Despite the tackling idea of the “magic probiotic”, leading scientists in the field have warned that first it is necessary to better characterise the local microbiota of the lung and its interaction with the host (Dickson and Cox 2017). Therefore, before passing to the development of interventional approach in human subjects based on previous studies in animals, it was necessary to further investigate and understand concomitant factors in the host’s response to lung cancer.

As the thesis project, we designed a clinical trial including NSCLC patients eligible for surgical resection of the tumour. Several objectives were set:

- (i) to characterise the gut, lung and upper airway microbiota in these patients,**
- (ii) to evaluate the homogeneity/heterogeneity between different microbiota within the same subject/group of patients, and**
- (iii) to evaluate the impact of the microbiota composition on immune and inflammatory status of the patient (evaluated in the gut, blood, lung).**

To meet these objectives, we included 18 patients and retrieved samples of blood, saliva, faeces and four lung samples (BAL, non-malignant tissue, peritumoural tissue and tumour) at hospital admission and during surgery. Saliva, faeces and four lung samples were used for DNA extraction and the analysis of microbiota by 16S RNA gene sequencing and qPCR. To limit the

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risk of contamination of BAL by upper airways, the washing was performed directly on the excised lobe. Also fresh faecal samples were used for microbiological enumeration of functional bacterial groups and frozen faeces was used for SCFA dosage. Immune status was evaluated by characterisation of the tumour infiltrating lymphocytes, phenotyping of the Th lymphocytes and neutrophil subtypes in BAL and blood, by cytokine dosage in blood and dosage of inflammatory and antibacterial markers in faeces. Each patient also provided one-week nutrition survey before intervention to be used as a help in interpreting other results (such as SCFA quantities). Other clinical and demographic information were also collected, such as living area, smoking history, or profession.

Our first hypothesis has been that the lung microbiota differs depending on the sampling location, even between samples in histological proximity, as peritumoural and tumour tissue. The samples chosen for analysis have different physiological functions and/or architecture, but also different interaction with the immune system. BAL represents microbial population of the bronchial lumen, therefore mostly bacterial population with planktonic characteristics, or on the contrary, associated to biofilms or mucus excreted by alveolar cells. Non-malignant tissue taken on the opposite lobe side from the tumour should represent a sample with normal lung architecture of small alveoli and single-layered lung epithelium, resembling to a “sponge”. Depending on the histological type, tumour could vary from highly dense tissue, as in SCC, to one enriched in mucus, as in certain types of ADK. Increased mucus production could directly serve as a nutriment for mucinolytic bacteria (Flynn et al. 2016) and favour the growth of certain bacterial groups. Finally, peritumoural tissue refers to the tissue surrounding the tumour separated based on differing histological characteristics. Even though by architecture peritumoural tissue resembles to non-malignant tissue, its proximity to tumour makes it exposed to various products excreted by tumour cells and to possible invasion of tumour cells themselves (Dou et al. 2018). Another important aspect is the role of the peritumoural tissue, i.e. the tumour microenvironment (TME), on direct tumour suppression or stimulation by different means, such as composition of the extracellular matrix, production of growth factors, etc. (Valkenburg, De Groot, and Pienta 2018). Both could directly (changes in tissue architecture) or indirectly (immune response against tumour cells) influence the local microbiota.

Next, we have hypothesised that there would be several factors potentially discriminating for both immune system and microbiota in the six samples (saliva, faeces and 4 lung samples). First is the histological type of the tumour, already reported to alter salivary microbiota of lung cancer patients (Yan et al. 2015) but also to change alpha diversity within tumour tissue (Yu et

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al. 2016). Except direct impact on microbiota as explained in the previous paragraph, different histological types are characterised by different immunogenicity, immunosuppressive nature and genetic background (Busch et al. 2016), meaning that they will not equally stimulate immune system. Consequently, influx and profile of the immune cells to the tumour bed will vary, and therefore also the interaction with the local microbiota. This genuine nature of the tumour and local immune status could reflect on systemic markers, and so on the intestinal immune status. Accordingly, gut microbiota could also vary between different tumour types. However, in this study type, it could not be discerned whether this change is a consequence of a disease, a concomitant factor or result of the change in the immune balance.

The second important factor is smoking status. Cigarette smoke has several undesirable effects on the respiratory system, such as inhalation of the hot air and precipitation of potentially carcinogenic particles. Both damage epithelial cells and impair airway clearance, inducing local and systemic inflammation (Çolak et al. 2019), therefore changing the local growth conditions of resident microbiota and disturbing the immune homeostasis. Since upper airways are first affected by the smoke, smoking-related changes in microbiota have already been reported (Wu et al. 2016) but interestingly, not in the lower airways (Segal et al. 2013). However, it is possible that not all microbiota of the lung is equally sensitive to smoking effects due to the discussed reasons. Therefore, this was one of the hypothesis to verify. Further, smoking was reported to alter gut microbiota both in healthy individuals (Stewart et al. 2018) and in lung cancer patients (Zhang et al. 2018; Zhuang et al. 2019), clearly showing the remote-site effect.

Mentioning the entry of the smoke from upper airways towards the lower ones brings us to another important factor – tumour location. Lungs are organised in five lobes, two on the left side (upper and lower) and three on the right side (upper, middle and lower). Interestingly, lower overall survival and worse prognosis were associated to tumours in lower lobes, and especially in the lower left lobe (Kudo et al. 2012). It has been previously suggested by the adapted island model of the lung biogeography from healthy subjects that lung microbiota is different between the lobes (Dickson et al. 2015). The model considers the principal bronchus as the source of the lung microbial communities, whose richness decreases proportionally to increase in the distance from the bronchus. Therefore, upper lobes should be richer than lower lobes, but contralateral lobes should have similar characteristics. However, the study that compares contralateral BAL (i.e. from the left and right lobe), one with cancer and one without it, shows that this is not true in lung cancer (Liu et al. 2018). It is clear here that tumour presence

Thesis objectives and hypotheses

modifies microbial composition, and that further studies including lung cancer patients are necessary.

We have concentrated on another important factor with crucial prognostic role in NSCLC treatment, the tumour staging. We have hypothesised that the tumour stage, withholding several important tumour characteristics as size and metastatic dissemination (TNM classification (Goldstraw et al. 2016)), will have an important impact on multiple parameters followed by this project. The higher the stage, the worse the overall prognosis for the patient. However, stage is determined by the combination of the three elements (T- tumour size, N – mediastinal and ipsilateral lymph nodes metastasis, M – distant metastasis) and can therefore sometimes represent genuinely different tumours. For example, a very large tumour without metastatic lymph nodes could belong to the same stage as a small tumour but with metastatic lymph nodes. For the purpose of the study of microbiota, we have decided to observe these elements separately and define the hypothesis that each of the elements could individually influence our microbial communities. Since patients with distant metastasis are not included in the study due to the difference in the treatment procedure (not operable), we considered other two elements – tumour size and metastatic lymph nodes. Depending on the diameter, tumour could be more or less oxygenated due to the often very leaky and insufficient vascularisation for larger tumours, with consequentially different degree of inner necrosis (Wouters et al. 2003). As discussed in the chapter 2.3.9.1 Egg or chicken, this could favour the colonisation of anaerobic bacteria that could either contribute to tumour progression with lactate production or inversely, increase the tumour immunogenicity (Baban et al. 2010; Damgaci et al. 2018; Van Dessel et al. 2015). Larger tumours could also block bronchial passages and create microaerobic or anaerobic pockets again favouring changes in microbial composition or pathogen colonisation. Metastatic lymph nodes are, on the other hand, a sign of higher tumour aggressiveness and a leaky vasculature enabling dissemination of the tumour cells to proximal lymph nodes regardless of the tumour size. This suggests in the same time the invasive nature of the tumour but also the “allowing” surroundings enabling the spread of tumour cells. According to Dr. Bissell (Bissell and Hines 2011), the tumour environment has a tremendous role in deciding tumour’s development and fate. In our concrete situation, this could refer to certain bacteria in the tumour environment that e.g. bind different cellular integrins cells (Garrett 2015; Khan et al. 2012), occupying the sites for metastatic diapedesis of tumour cells. Since presence of metastatic lymph nodes has been important negative prognostic marker for overall survival regardless of tumour size (Liu, Chen, and Xu 2017), it has been prioritised in our analysis.

Thesis objectives and hypotheses

It is known that cancer patients, especially with more advanced cancer stages, often report weight loss without particular change in diet. This phenomenon called cachexia is explained by voracious cancer cells that can uptake the most of the host's energy obtained through digestion, or by different cytokines produced by the host as a response to cancer that modify host's metabolism (Barton 2001). Changes in the gut microbiota related to presence of cancer, as well as application of probiotics in fighting cachexia have been considered but the current conclusion insists on further research (Bindels and Thissen 2016). Therefore, our hypothesis considering the effect of tumour stage on lung microbiota was extended also to the gut microbiota, accompanied with the patient's documentation on weight stability and nutritional survey for personalised approach.

Finally, the theory of the gut-lung axis implicates that certain elements with intestinal origin, such as bacteria or their products, prime immune cells situated in mesenteric lymph nodes. Those are afterwards recruited to distant sites where they could exert improved efficacy (Bingula et al. 2017). In our case, this could be characterised by an improved lung tumour infiltration with immune cells. However, it is important to keep in mind that there are three general tumour immune types; immune-inflamed, immune-excluded and immune-desert tumours (Chen and Mellman 2017). Simplified, immune-inflamed tumours are well infiltrated and immunogenic, while immune-desert tumours are poorly infiltrated and poorly immunogenic. Finally, immune-excluded tumours are often immunogenic but with certain intrinsic element, such as elevated expression of PD-1, that inhibits immune cell infiltration and activity. Intuitively, one type could pass to another, for example immune-inflamed tumour that after intervention of the immune system keeps only low immunogenic cells could become immune-desert. Nevertheless, inverse is also possible. Better priming of the immune cells by e.g. inducing cross-reactivity between tumour cells and bacterial epitopes, could turn an immune-desert tumour to immune-inflamed. Therefore, our hypothesis has been that gut, but also lung microbiota, will vary depending on the immune type of the tumour.

To summarise, the discussed hypotheses include only the initial, principal hypotheses that will be further developed with the progress in analyses. The major hypotheses mostly include factors with both local and systemic influence on the host, and therefore to its microbiota and immune system. This was the underlying reason to approach patients from different angles (microbiota from three sites, local and systemic immune and inflammatory markers by different techniques, bacterial products, nutrition, and demographic data). The objectives and hypotheses answered at the moment will be discussed in the chapter "Results" and "General discussion".

Materials and methods

Article: Study protocol

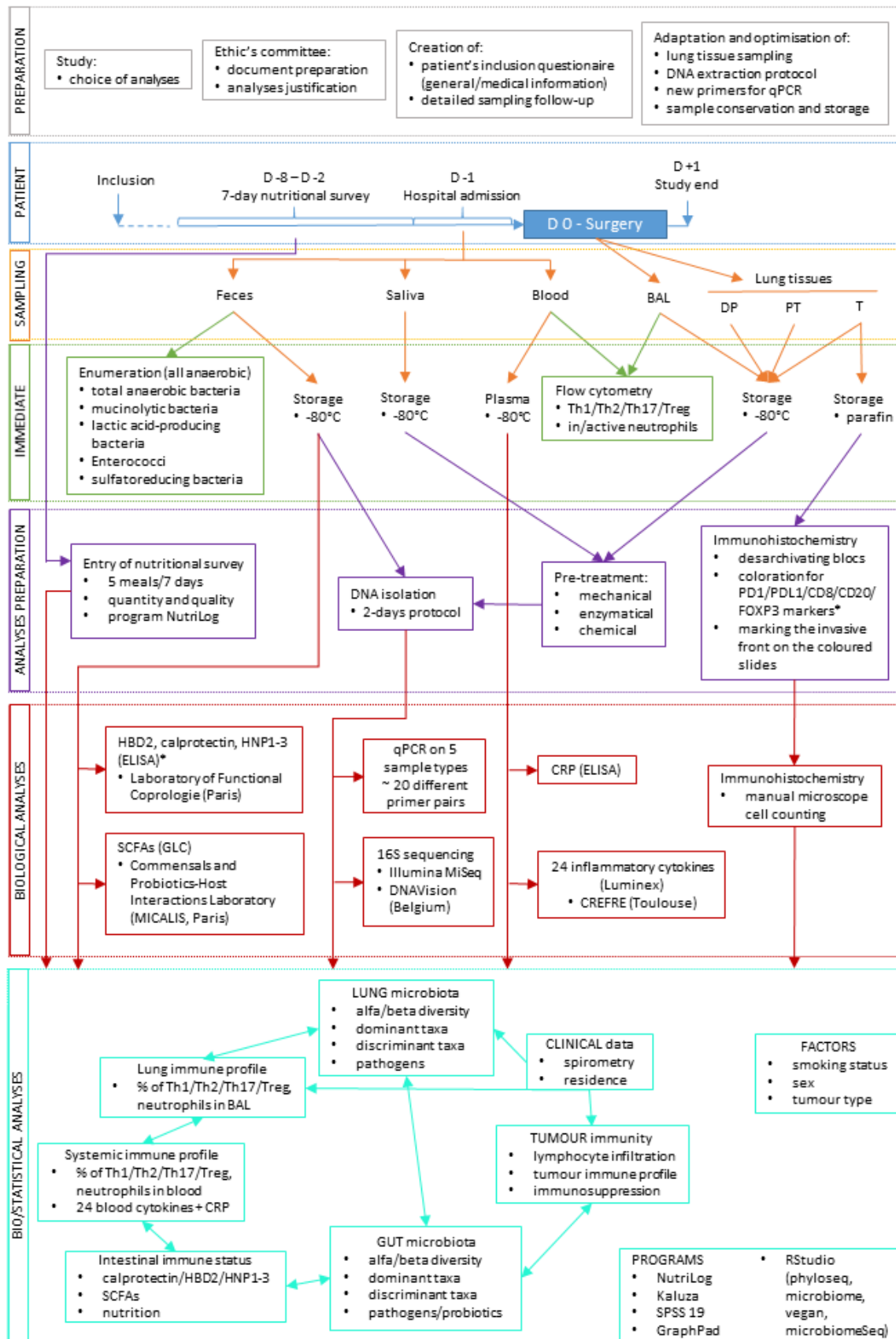
Study Protocol

Characterisation of Gut, Lung and Upper Airways Microbiota in Patients with Non-Small Cell Lung Carcinoma: Study Protocol for Case-Control Observational Trial

Rea Bingula, MS,¹ Marc Filaire, Prof, MD,^{1,2} Nina Radosevic-Robin, MD,³ Jean-Yves Berthon, PhD,⁴ Annick Bernalier-Donadille, PhD,⁵ Marie-Paule Vasson, Prof,^{1,6} Emilie Thivat, PhD,^{7,8} Fabrice Kwiatkowski, MS,^{7,8} Edith Filaire, Prof^{1,4}

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The majority of the studies exploring the lung microbiota by high-throughput sequencing use commercially available kits for microbiota isolation that are not specifically designed for lung samples (e.g. MoBio PowerSoil or Qiagen's QIAampStoolMinikit). Since lung samples differ significantly in quality (very fibrous tissue) and bacterial number from faeces or soil, these kits might not be the best adapted nor with the optimal DNA yield (filtration columns, etc.). Therefore, we have decided to create a "lung microbiota isolation" protocol, based on HMP protocol for gut microbiota isolation and previously compared in performance with other commercially available kits (Costea et al. 2017; McInnes 2010). The final optimised version of the protocol, including the study design, all used materials and methods have been published under the running title "Characterisation of gut, lung, and upper airways microbiota in patients with non-small cell lung carcinoma". Only the part of the project considering patients eligible for surgery without chemotherapy has been considered in this thesis manuscript, and its synthetic overview is shown in the Figure 9.



BAL – Bronchoalveolar lavage fluid; CRP – C-reactive protein; D – day; DP – healthy lung tissue; GLC – gas-liquid chromatography; ELISA – enzyme-linked immunosorbent assay; HBD2 – human β defensin 2; HNP – human neutrophil protein; PT – peritumoral tissue; SCFA – short-chain fatty acids; T – tumour

Figure 9 Synthetic overview of the study protocol

Study Protocol

Characterisation of Gut, Lung and Upper Airways Microbiota in Patients with
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Observational Trial

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Abstract

Background. Several studies have confirmed the important role of the gut microbiota in the regulation of immune functions and its correlation with different diseases, including cancer. While brain-gut and liver-gut axes have already been demonstrated, the existence of a lung-gut axis has been suggested more recently, with the idea that changes in the gut microbiota could affect the lung microbiota, and *vice versa*. Likewise, the close connection between gut microbiota and cancer of proximal sites (intestines, kidneys, liver, etc.) is already well established. However, little is known whether there is a similar relation when looking at world's number one cause of death from cancer – lung cancer.

Objective. Firstly, this study aims to characterise the gut, lung and upper airways (UAs) microbiota in patients with non-small cell lung cancer (NSCLC) treated with surgery or neoadjuvant chemotherapy plus surgery. Secondly, it aims to evaluate a chemotherapy effect on site-specific microbiota and its influence on immune profile. To our knowledge, this is the first study that will analyse multi-site microbiota in NSCLC patients along with site-specific immune response.

Methods. The study is a case-controlled observational trial. Forty NSCLC patients will be divided into two groups depending on their anamnesis: (i) Pchir, patients eligible for surgery, or (ii) Pct-chir, patients eligible for neoadjuvant chemotherapy plus surgery. Composition of the UAs (saliva), gut (faeces) and lung microbiota (from broncho-alveolar lavage fluid (BALF) and three lung pieces: “healthy” tissue distal to tumour, peritumoural tissue and tumour itself) will be analysed in both groups. Immune properties will be evaluated on the local (evaluation of the tumour immune cell infiltrate, tumour classification and properties, immune cell phenotyping in BALF; human neutrophil protein (HNP) 1-3, β -defensin 2 and calprotectin in faeces) and systemic level (blood cytokine and immune cell profile). Short-chain fatty acids (SCFAs) (major products of bacterial fermentation with an effect on immune system) will be dosed in faecal samples. Other factors such as nutrition and smoking status will be recorded for each patient. We hypothesise that smoking status and tumour type/grade will be major factors influencing both microbiota and immune/inflammatory profile of all sampling sites. Furthermore, due to non-selectivity, the same effect is expected from chemotherapy.

Abbreviations:

ANSM - The French National Agency for Medicines and Health Products Safety (*Agence nationale de sécurité du médicament et des produits de santé*); BAL - broncho-alveolar lavage;

BALF – broncho-alveolar lavage fluid; BMI – body mass index; CIFRE - Industrial Research Training Agreements grant (*Convention industrielle de formation par la recherche*); CRP – C-reactive protein; CT – chemotherapy; ELISA – enzyme-linked immunosorbent assay; FDR – false discovery rate; GF – germ-free; GI – gastrointestinal; HBSS – Hank’s balanced salt solution; HNP – human neutrophil peptide; ICI – immune checkpoint inhibitor; IL – interleukin; MCLB – mammalian cell lysis buffer; NSCLC – non-small cell lung cancer; OTU – outer taxonomic unit; Pchir – patient surgery, fr. “patient chirurgie”; Pct-chir – patients chemotherapy plus surgery, fr. “patient chimiothérapie – chirurgie”; qPCR – quantitative polymerase chain reaction ; SCFAs – short-chain fatty acids; UAs – upper airways.

1 Introduction

The microbiota is a consortium of different microorganisms that includes bacteria (microbiota), fungi (mycobiota), viruses and protozoa residing on the skin and in the oral, pulmonary, urogenital and gastrointestinal (GI) cavities, with the GI tract having the highest density of microorganisms. The functional importance of the microbiota to the host is undeniable, involving functions that range from the breakdown of complex dietary polysaccharides to competing with pathogens and modulating the mucosal and immune system in general.¹ Gut dysbiosis is now considered to be an underlying cause of a wide range of GI diseases and an emerging number of non-GI conditions such as obesity and cardiovascular disease, as well as a range of psychiatric diseases.² Recently, an emerging number of studies began to address the relation between gut microbiota and the lung. This relation has been referred to as the “gut-lung axis”. The basis of this axis theory lies in the “gut-lymph” theory of Samuelson *et al.*³ The theory says that the large numbers of macrophages and other immune cells are present in the intestinal submucosa or mesenteric lymph nodes, where the majority of translocating bacteria are also found. If not eliminated by this first line defence, surviving bacteria, cell wall fragments or the protein fractions of dead bacteria escape with the cytokines and chemokines produced in the gut, travel along the mesenteric lymphatic system to the cisterna chyli, and subsequently enter the circulatory system. Thereby they have access to pulmonary circulation, which may lead to the local activation of dendritic cells and macrophages as well as T cell priming and differentiation. Another way to influence the pulmonary region might be through the migration of immune cells themselves, after priming and activation at the first site of antigen encounter, i.e. the gut mucosa. Although this theory explains the unilateral interaction, it is reasonable to speculate that this axis works the same way when it originates in the lung mucosa and lung lymph nodes.⁴ Moreover, nutrition can also affect both immune response and composition of

our respiratory tract microbiota.⁵ In mice, high-fibre diet increased protection against allergic inflammation in the lung (reduced inflammatory cell infiltration), followed by a change in the gut and, to a lesser extent, the airway microbiota.⁶ The study also reported an increase in blood levels of circulating SCFAs, one of the major products of bacterial fermentation responsible of intestinal barrier integrity and known for its anti-inflammatory properties. However, no traces were found in the lung itself. On the contrary to allergic inflammation, a lack of an appropriate stimulus during the developmental phase of an immune response, as during infection, will disable a quick and effective immune reaction. This could result in undesirable consequences such as pathogen colonisation, increased susceptibility to infection, tissue damage, possible development of cancer and increased mortality.^{7,8} Therefore, it is clear that there is a complex network of distinct and precise stimuli that are required for executing a correct immune response. According to the gut-lung axis theory, these stimuli can originate in the gut, explaining the observed protective effect in the lung. Taking a huge step forward, the study of Routy *et al.* (2018)⁹ evaluated the role of gut microbiota in responsiveness to anticancer treatment by immune checkpoint inhibitors (ICI) (PD-1/PD-L1). They showed that non-small cell lung cancer (NSCLC) patients that received antibiotic treatment (ATB) during 2 months prior to therapy had significantly decreased overall and progression-free survival. Similarly, ATB treatment was a predictor of ICI resistance, independent from other prognostic markers. When faecal microbiota transfers using the stool from NSCLC patients responding or not responding to therapy were performed, inoculated germ-free (GF) mice showed the same phenomenon during ICI therapy against MCA-205 tumours. Mice receiving ICI therapy that were inoculated with a responder's stool showed delayed tumour growth and accumulation of antitumour lymphocytes in the tumour microenvironment. The stool of NSCLC patients responding to ICI therapy was found to be enriched in phylum *Firmicutes*, as well as distinct genera such as *Akkermansia*, *Ruminococcus*, *Alistipes*, etc. In further experiments with GF mice, *Akkermansia muciniphila* proved to be sufficient to restore ICI therapy responsiveness, both when inoculated alone or with the stool from non-responding NSCLC patients, rectifying the response. Likewise, it was the only species that induced reactivity from patient-derived Th1 and Tc1 *in vitro*, and that correlated with progression-free survival. Looking at these results, it is evident that the gut microbiota plays a crucial role in the host's homeostasis and that its fine-tuned composition counts for much more than was previously thought. However, data on this topic remain scarce but directed to a promising field of a new anti-lung cancer approach that the world population is yearning for.¹⁰ Unlike the local and systemic influence of the gut

microbiota, the influence on and of the lung microbiota and its products has yet to be properly assessed, both in health and disease.¹¹

Therefore, to help to elucidate this new and extremely interesting field, we have decided to conduct a case-control observational study in patients with NSCLC. The study will include two groups of patients: i) group Pchir, with patients eligible for treatment by surgery, and ii) group Pct-chir, with patients eligible for a combined treatment consisting of neoadjuvant platinum-based chemotherapy followed by surgery.

The objectives of this study are:

- (i) to characterise the gut, lung and upper airway microbiota in these patients;
- (ii) to evaluate the homogeneity/heterogeneity between different microbiota within the same subject/group of patients;
- (iii) to evaluate the impact of the microbiota composition on immune and inflammatory status of the patient (evaluated in the gut, blood, lung);
- (iv) to evaluate the effect of chemotherapy on the site-specific microbiota (UAs, lung, gut).

While group Pchir will have only one time point for sample collection, group Pct-chir will have multiple time points. The latter will enable follow-up on changes in microbiota and immune markers relative to the treatment progression.

2 Methods and Analyses

2.1 Ethics approval and dissemination

This protocol has been approved by the Committee for the Protection of Persons (CPP) Sud-Est VI, Clermont-Ferrand, France, and The French National Agency for Medicines and Health Products Safety (ANSM) (study ref. 2016-A01640-51). Because of the invasiveness of the sampling techniques, the requested control group was not approved by the CPP. The study was accompanied by amendment approved by ANSM in June 2018. The current protocol is entitled “Protocol MICA V3”, and presents an up-to-date version and the version in use. This study is registered with the Clinical Trials under ID: NCT03068663. The study’s official name is: Characterisation of the microbiota (gut, lung and upper airways) in patients with non-small cell lung carcinoma: exploratory study (acronym: MICA). Written informed consent is obtained from all patients before enrolment in the study. The results are planned for presentation at conferences and publication in peer-reviewed journals in early 2019. All samples will be

preserved for 15 years according to the practice of the sponsoring institution (Centre Jean Perrin). Samples will be available to other investigators if they want to perform complementary studies that consider NSCLC after additional consent obtained from patient. However, because of French regulations regarding patient information files, patients' data will not be available.

2.2 Study outcomes

As its primary outcome, this study will characterise the lung and UAs microbiota in two groups of 20 patients with NSCLC. Group Pchir will include patients eligible for surgery without chemotherapy. Group Pct-chir will include patients eligible for surgery after platinum-based chemotherapy. UAs microbiota will be evaluated from saliva, while lung microbiota will be evaluated from (i) three lung explants: "healthy" lung tissue, tumour and peritumoural tissue, and (ii) broncho-alveolar lavage fluid (BALF) from the tumour's proximity (same lobe). Following bacterial DNA extraction, microbiota will be analysed by qPCR and 16S ribosomal rRNA gene sequencing using the Illumina MiSeq platform.

Secondary outcomes of this study are set as follows:

- i) to study the effect of neoadjuvant chemotherapy on microbiota by evaluating:
 - a. the variation of the proportion of the phylum *Firmicutes* (as the phylum is highly represented in all of the different types of samples considered in this study),⁴
 - b. the variation of the proportion of the bacterial genera per phylum in different types of samples (faeces, saliva, BALF, lung tissue/peritumoural tissue/tumour),
 - c. the concordance of genera between sample locations (e.g. saliva vs. BALF, healthy lung tissue vs. peritumoural tissue vs. tumour);by qPCR and 16S rRNA gene sequencing;
- ii) to study the homogeneity/heterogeneity between lung, upper airways and gut microbiota for each and between both groups (evaluated by 16S rRNA gene sequencing and qPCR) and between different time points (group Pct-chir),
- iii) to evaluate immune/inflammatory status:
 - a. in the gut: by dosing β -defensin 2, human neutrophil peptides HNP1-3, calprotectin (ELISA) and SCFAs (gas liquid chromatography)
 - b. in plasma: by dosing plasmatic cytokines (Luminex), C-reactive protein (CRP) (ELISA) and immune cell phenotyping (flow cytometry)

- c. in the lung: by immune cell phenotyping (flow cytometry) and characterisation of immune infiltrate in lung tumour biopsies obtained during the operation (by immunohistochemistry).

2.3 Patient recruitment

The study pre-considers all patients diagnosed with NSCLC and presented before the Thoracic Oncologic Committee of the Jean Perrin Centre, Clermont-Ferrand, France. Inclusion criteria are presented in Table 1. Inclusion in the study is consecutive and parallel for both groups. A written informed consent is obtained from each patient participating in the study before inclusion. Depending on their diagnosis, patients are included in one of the two groups: Pchir (patients eligible for surgery only), or Pct-chir (patients eligible for surgery after neoadjuvant platinum-based chemotherapy).

Table 1 Inclusion and exclusion criteria for patients

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> • NSCLC patient with an indication of surgery or neoadjuvant chemotherapy plus surgery • >18 and <80 years of age • BMI <29.9 • not-treated with antibiotics, corticoids or immunosuppressive drugs for at least the past 2 months • signed written consent before enrolment in the study • affiliated with the Social Security System 	<ul style="list-style-type: none"> • cognitive difficulties • refusal or inability to give clear consent to participate • acute digestive or pulmonary infections in the past 2 months (requiring antibiotic treatment) • inflammatory intestinal pathologies • colostomy • partial or complete gastrectomy • previous oesophageal surgery • previous otorhinolaryngeal cancer treated by radiotherapy or surgery • inability to conform to the study's requirements • deprivation of a right to decide by an administrative or juridical entity • ongoing participation or participation in another study <1 month ago • ground-glass opacity

2.4 Patient and public involvement

Neither patients nor the Patient Committee were involved in the design of this study. If desired, patients can be informed of the study's results by the investigator or physician.

2.5 Trial design and timeline

This study is a case-control observational trial. Recruitment into the study started in May 2017 and will end in May 2019. The estimated complete duration of the study is 29 months, with an average follow-up period per patient of 1.5 and 3.5-4.5 months for the Pchir and Pct-chir groups, respectively. Intermediary analyses will take place after obtaining all samples from half of the patient quota ($n = 20$) regardless of the group, without interruption of the further recruitment to the study.

The trial design is presented in Figure 1. Eligible patients meet official study personnel at an outpatient appointment (Visit 1) where all the details of the protocol are thoroughly explained. At this visit, patients give their written consent to participate in the study. Depending on their clinical diagnosis, they are assigned to one of the two groups (Pchir or Pct-chir). Each patient is given a protocol summary, sampling instructions and corresponding number (depending on inclusion group) of tubes for saliva, boxes for faecal samples with anaerobic atmosphere generation bags and templates for a 7-day nutritional survey. Other information is also recorded, such as smoking status and history, weight or diet modifications in the last several months/years, cohabitation or interaction with animals in their childhood/present, the environment in which patients grew up/spent their life (countryside/city), exposure to certain pollutants, e.g. related to their profession, etc. This information will serve for better understanding of the individual's inflammatory status and microbial characteristics.

As presented in Figure 1, group Pchir has sampling concentrated around one event – surgery. At hospital admission (D-1 before surgery), samples of saliva, blood and faeces are collected together with the nutritional survey patients made during the preceding week (D-7 to D-1). BALF and lung tissue samples are collected during the surgery (D0) from the excised lobe.

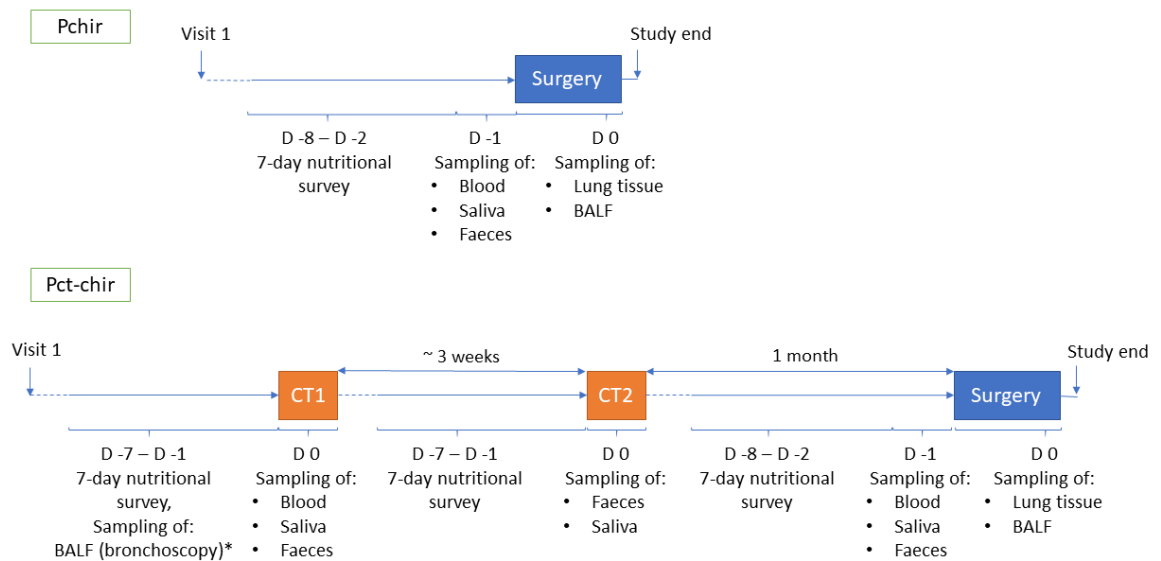


Figure 1 Study flowchart. BALF – broncho-alveolar lavage fluid; CT – chemotherapy; D – day; Pchir – the group of patients undergoing surgery; Pct-chir – the group of patients undergoing chemotherapy and surgery. *performed as a part of patient’s standard medical care if decided by his/her physician.

Group Pct-chir (Figure 1) has sampling concentrated around three events:

- i) 1st chemotherapy cycle (CT1) - the day of CT1, samples of saliva, blood and faeces are collected together with nutritional surveys patients made during the preceding week (same as D-1 for Pchir group). After sample retrieval, patients continue with their medical care procedure. Optionally, in the days preceding the CT1 as the part of their standard medical care, patients can undergo bronchoscopy. In this case, a part of retrieved liquid (BALF) is taken for microbiota characterisation and immune cell profiling.
- ii) 2nd chemotherapy cycle (CT2) - CT2 is usually 3 weeks after CT1. The day of CT2, only saliva, faeces and nutritional surveys are retrieved. After sample retrieval, patients continue with their medical treatment.
- iii) Surgery - The time elapsed between CT2 and surgery is about 1 month, depending on the patient’s overall health status. The sampling for surgery is exactly the same as for group Pchir. At hospital admission (D-1 before surgery), samples of saliva, blood and faeces are collected together with nutritional surveys patients made during the preceding week (D-7 to D-1). BALF and lung tissue samples are collected during the surgery (D0) from the excised lobe.

The strong point of this trial is that study participation does not modify the patient's standard care treatment in any way. Invasive intervention, such as sampling of the lung tissue, is done during operation on the lung already removed from the patient. BALF sampling during operation is performed directly on the dissected lobe, posing no additional inconvenience for the patient and drastically minimising UAs contamination, which is an important advantage to this study's concept. Likewise, sampling of the BALF at CT1 for group Pct-chir is done in the scope of a standard care procedure by bronchoscopy and it is not imposed by the study. However, the latter is also an inconvenience because there will be patients from group Pct-chir who will not undergo bronchoscopy with BALF sampling since it is not prescribed by his/her physician. Another thing to consider is also the difference in sampling technique of BALF. Sampling of BALF at CT1 for group Pct-chir is done by bronchoscopy, while sampling during surgery is done directly on the excised lobe for both groups. Therefore, the first BALF has a higher risk of contamination by UAs, while the second BALF (during operation) should have no UAs contamination and better represent luminal microbiota specific of the tumour lobe. This will be taken into account during data interpretation, and if necessary, each time point will be characterised for itself.

In conclusion, in the group Pct-chir, the effect of chemotherapy on the microbiota of saliva and faeces and immune parameters of blood and faeces will be systematically assessed. Chemotherapy's effect on immune parameters and microbiota in BALF will be assessed only if obtained data in quantity and quality will permit it (as explained above). When talking of lung tissue, "healthy" lung tissue, taken at distance from the tumour, will be considered as a control¹² tissue for comparison with peritumoural and tumoural tissue, as well as with BALF. Also, characterisation will be done respective to the tumour type where possible (sufficient patient number with the same tumour type).

A demanded control group for the study was not authorised by the Ethics Committee, due to the invasiveness or evident inability to realise certain sampling steps (bronchoscopy, lung tissue sampling). Therefore, the focus will be on characterisation of the site-related microbiota and its connection to local and systemic immunity in NSCLC respective of the treatment group (Pchir or Pct-chir). Likewise, the question is whether there is an initial difference between group Pchir and group Pct-chir (before CT1), and what its nature is. Equally, how does chemotherapy modify group Pct-chir, and whether it becomes more alike or different from group Pchir regarding its different properties (microbial taxa ratios, inflammatory properties) when followed in time (from CT1 to surgery). Furthermore, obtained results

considering microbiota composition and abundance, where possible, will address similar studies^{13–16} only in a descriptive matter and the same will be done with immune parameters.^{17–21}

2.6 Sampling and data recording

2.6.1 Nutritional survey

The dietary habits are evaluated for each patient for the 7 days preceding chemotherapy (Pct-chir) and/or surgery (Pchir and Pct-chir). At Visit 1 (see Figure 1), all participants receive a detailed verbal explanation, written instructions and the survey with an example. They are asked to maintain their usual dietary habits during the survey period and to record as accurately as possible the amount, type and preparation of food and fluid consumed. If they consume commercial and ready meals, they are also asked to note brand names. The quantity of food or drink can be expressed in either precise measures (weight) or in common household measures, such as cups, tablespoons, etc. In the case of any questions or ambiguities, patients are encouraged to contact the study personnel. These data will help to estimate each patient's overall nutritional status and help to explain the microbiological analysis of faecal samples following the survey, as well as the patient's immune and inflammatory status. We anticipated that the patients might change their dietary habits during the chemotherapy duration, which is why recording was requested before each chemotherapy treatment and surgery, and during one week. The primary objective is to use what was recorded as complementary data to the faecal microbiota analysis (sampled the day after the end of each survey), to better explain the longitudinal modification of microbiota, if any. This is due to the fact that faecal microbiota can overcome significant changes in only a few days relative to a diet change.²²

2.6.2 Saliva

At Visit 1, after recording any evidence of oral health problems or injuries, each patient receives a tube for saliva collection (Sarstedt). It is necessary to fill the tube with a minimum of 1 mL of saliva (designated on the tube) on an empty stomach by the passive drooling method on the morning of hospital admission for the chemotherapy session (Pct-chir) and/or surgery (Pchir, Pct-chir) (Figure 1). The sample is stored at -80°C for later bacterial DNA extraction and metagenomic sequencing.

2.6.3 Blood

Blood sampling is done following the patient's admission to hospital, in the day/hours preceding the prescribed clinical treatment, depending on the group (Figure 1). 10 mL of fresh

blood are collected in EDTA treated tubes, where 500 μ L of whole blood is immediately used for immune cell phenotyping by flow cytometry. The remainder is centrifuged for 10 min 2,000 x g at 4°C to obtain plasma, which is then aliquoted and stored at -80°C for further analyses (cytokine/interleukin analysis by Luminex, CRP dosage by ELISA).

2.6.4 Faeces

At Visit 1, each patient receives a sampling box, an anaerobic atmosphere generation bag (GENbag anaer, Biomérieux) and detailed printed instructions on how to handle the samples at his/her home. Faecal samples are collected following the 1-week nutritional survey (i.e. on the day of chemotherapy before the drug infusion or the day preceding surgery). In brief, sampling is done directly into the sampling box, and after removing the protective foil and placing the anaerobic atmosphere generation bag in the box, the box is closed firmly and placed in the cold (+ 4°C). Patients are asked if they have the ability to transport the sample in an insulated bag to preserve cold conditions. If there is no such possibility, the study personnel supplies the patient with the requested bag. The sampling should be done within 12 hours preceding the hospital admission and sample retrieval. Therefore, patients are asked to do the sampling the morning of hospital admission if possible. If there are any problems, the patient is asked to contact the protocol personnel to ensure that the sample is processed in time. On reception, the sample is aliquoted 3 x 1 g for bacterial DNA extraction, and 2 x 5-10 g (depending on availability) for dosage of faecal calprotectin, β -defensin 2, HNP1-3 (ELISA) and SCFAs (gas liquid chromatography). Aliquots are stored immediately at -80°C until analysis. Approximately 1 g of fresh sample is used for bacterial culture of the main functionary groups of microorganisms (total anaerobic bacteria, mucin-degrading bacteria, lactic acid producing bacteria, sulphate-reducing bacteria and *Enterobacteriaceae*).

2.6.5 Lung tissue and BALF

Only for group Pct-chir, BALF is sampled at inclusion to the study. This sample is taken as a part of patient's standard care protocol if decided by his/her physician, and is not taken as an additional sample for this study. BAL is performed by routine bronchoscopy procedure.

Sampling of lung tissue and BALF during surgery is performed for both groups, after partial or complete pneumonectomy. The removed lung tissue is placed in a sterile vessel and the tumour position is determined by palpation. A piece of healthy lung distal to the tumour, with a minimum size of 1 cm x 1 cm x 1 cm, is then clamped. The clamp is left in place during the following procedure. The stich on the bronchus is cut away and using a sterile syringe the lung

is inflated through the bronchus. Lavage is performed by instilling 2 x 40 mL of sterile physiological saline. After each instillation, the maximum amount of liquid inside the bronchus is retrieved (8-10 mL in total), poured into a sterile 50 mL tube and placed immediately on ice, designated as “BALF”. At the end, the clamped wedge is cut off and designated as “healthy lung”. A slice of the tumour, with a minimal weight of 400 mg, containing the tumour cross-section is excised along with peritumoural tissue, after which the two are separated based on histological difference. All tissues are frozen first in liquid nitrogen and then placed at -80°C for long-term storage until DNA extraction. The mirror piece of the excised tumour slice is stored in paraffin and later analysed by immunohistochemistry for characterisation of tumour infiltrate. 3 mL of BALF are immediately used for immune cell analysis by flow cytometry and the remainder is stored at -80°C for later DNA extraction.

2.7 Methods and Analyses

Saliva, “healthy” lung, peritumoural and tumour tissue, BALF and faecal samples will be used for bacterial DNA extraction, followed by qPCR and 16S rRNA gene sequence analysis to establish microbial profiles. Fresh faeces samples will be used for bacterial culture, and frozen aliquots for dosage of faecal calprotectin, β -defensin 2, HNP1-3 (ELISA) and SCFAs (gas liquid chromatography). BALF and plasma samples will both be analysed by flow cytometry (immune cell phenotyping), while plasma will also be used for cytokines (Luminex) and CRP (ELISA) dosage. Tumour tissue stored in paraffin will be used for the analysis of immune infiltrate by immunohistochemistry. Each procedure is explained in detail in the following sections.

2.7.1 Nutritional status

Nutritional data for each patient will be analysed using the Nutrilog 2.3 software package, a computerised database (Proform) that calculates food composition from the French standard reference.²³

2.7.2 Cytokines

Stored plasma will be analysed for cytokines corresponding (but not restricted) to the following profiles: Th1, Th2, Th17, Treg. Samples will be analysed using Luminex kits: HSTCMAG-28SK, HTH17MAG-14K and TGFBMAG-64K-01 (Merck Millipore). Analyses will be conducted by the phenotyping service of CREFRE, Toulouse, France.

2.7.3 Evaluation of tumour immune infiltrate

Immunohistochemistry will be performed on tumour tissue using the specific antibodies to detect subpopulations of immune cells as follows: cytotoxic T-lymphocytes (anti-CD8, clone SP16, ThermoFisher Scientific), regulatory T-lymphocytes (anti-FoxP3, clone SP97, ThermoFisher Scientific), B-lymphocytes (anti-CD20, clone SP32, Cell Marque). The immune response checkpoint axis PD-1–PD-L1 will be assessed by anti-PD-1 (clone NAT105, Cell Marque) and anti-PD-L1 (clone 28-8, Abcam). All staining will be performed by a fully automated, standardised procedure (Benchmark XT, Ventana/Roche). The number of lymphoid cells expressing each antigen, except PD-L1, will be determined within 5 consecutive x40 microscopic fields, starting from the invasive front toward the tumour centre, used as a parameter reflecting the tumour's quantity of a given immune cell subpopulation. PD-L1 will be assessed for both immune and tumour cells and reported as the percentage of each population expressing the antigen.

2.7.4 Immune cell phenotyping

Immune cell phenotyping will be performed on fresh samples of blood (0.5 mL) and BALF (3 mL) by flow cytometry. Leukocytes will be obtained after haemolysis (solution of 155 mM NH_4Cl , 12 mM NaHCO_3 , 0.1 mM EDTA) for 15 min at room temperature, followed by 10 min centrifugation at 600 x g. Before centrifugation, BALF will be filtered through a porous gauze to eliminate mucus and reduce the viscosity. Lymphocyte subpopulations will be phenotyped using the following antibodies: anti-CD3-VioBlue, anti-CD4-APC-Vio770, anti-CD25-APC, anti-CD127-VioBright FITC, anti-CD183 (CXCR3)-PE-Vio770, anti-CD294 (CRTH2)-PE, anti-CD196 (CCR6)-PE-Vio615, anti-CD15-FITC, anti-CD62L-PE, anti-CD11b-PE-Vio770 and Viability 405/520 fixable dye, all purchased from Miltenyi Biotec. T lymphocytes CD4^+ will be characterised as $\text{CD3}^+\text{CD4}^+$ cells. Subpopulations of T lymphocytes CD4^+ will be characterised as follows: Th1 as $\text{CD3}^+\text{CD4}^+\text{CD183}^+$, Th2 as $\text{CD3}^+\text{CD4}^+\text{CD294}^+$, Th17 as $\text{CD3}^+\text{CD4}^+\text{CD196}^+$, Treg as $\text{CD3}^+\text{CD25}^+\text{CD127}^-$ and neutrophils as $\text{CD15}^+\text{CD11b}^+\text{CD62L}^+/\text{CD15}^+\text{CD11b}^-\text{CD62L}^+$ for “tethering” form, and $\text{CD15}^+\text{CD11b}^+\text{CD62L}^-$ for active form. Due to high debris background in BALF samples, utilisation of the Viability dye is essential and utilisation of intracellular dyes is excluded. The data will be acquired using LSRII, BD Biosciences.

2.7.5 Inflammatory/antimicrobial markers and short chain fatty acids (SCFAs) analysis

Faecal samples will be analysed for calprotectin (kit Calprest NG, Eurospital, with an adaptation on BEP2000 (Siemens)), β -defensin 2 (β Defensin 2 ELISA Kit, Immundiagnostik, Bensheim), and HNP1-3 (human HNP1-3 ELISA Kit, Hycult biotech). All three markers will be measured in the Laboratory of Functional Coprologie, GH Pitié-Salpêtrière, Paris. C-reactive protein will be measured in plasma by CRP human ELISA kit (Enzo Life Sciences). SCFA concentration will be dosed after water extraction of acidified faecal samples using gas liquid chromatography (Nelson 1020, Perkin-Elmer) in the Commensals and Probiotics-Host Interactions Laboratory, Micalis Institute, INRA UMR 1319, France.

2.7.6 DNA extraction

DNA extraction on all samples will be performed in batches to reduce the possibility of manipulation errors between extractions.

a) Sample pre-treatment

Lung tissue. Lung tissue will be taken directly from liquid nitrogen, broken into smaller pieces with a mortar and pestle, and homogenised in Hank's balanced salt solution (HBSS) (Sigma-Aldrich) in gentleMACS M tubes (Miltenyi Biotec). The ratio of buffer volume:sample weight will be determined for each sample, and adapted volume will be used for each of the following steps. The programs used will be those adapted for lung and tumour tissue (Miltenyi Biotec). The homogenate obtained will be treated with collagenase D (Sigma-Aldrich) (2 mg/mL final concentration) at 37°C for 15 min, followed by 10 min at 2,000 x g at room temperature (RT). The pellet will be resuspended in 2-5 mL (depending on the initial sample weight) of mammalian cell lysis buffer (MCLB),²⁴ and repeatedly vortexed for 5 min at RT. The reaction will be stopped with adding an equal volume of neutralisation buffer.²⁴ After two washes with PBS (Sigma) (2,000 x g for 10 min), the pellet will be used for DNA extraction using the adapted protocol of Godon *et al.*²⁵

Saliva and BALF. Saliva and BALF will first be brought to RT and vortexed. 1 mL of saliva and 5 mL of BALF will be used for DNA extraction. Whole BALF will be used for extraction, to minimise the loss of bacterial communities.²⁶ BALF will be centrifuged (7,000 x g, 10 min) and 3 mL of MCLB will be added to the pellet, while saliva will be treated directly with 1 mL of MCLB. Both will be vortexed for 5 min at RT, followed by addition of neutralisation buffer.

DNA extraction will be performed directly on the pellet after centrifugation at 7,000 x g for 10 min at RT and a washing step (PBS).

Faeces. Faecal samples will have no pre-treatment and extraction will begin directly on frozen samples.

b) DNA extraction

DNA extraction will be performed by using the adapted protocol of Godon *et al.*,²⁵ i.e. International Human Microbiome Standards Standard Operating Protocol for Fecal Samples (IHMS SOP) 07 V1. Briefly, 4 M guanidine thiocyanate and 10% N-lauroyl sarcosine will be added directly on frozen samples or pellets for 10 min at RT. After the addition of 5% N-lauroyl sarcosine and homogenisation by vortexing, the samples will be incubated for 1 hr at 70°C. All of the samples will be transferred to Lysing Matrix B tubes (MPBio) and homogenised using FastPrep®-24 Instrument (MPBio), 4 x 45 s at 6.5 m.s⁻¹. Between each cycle, the samples will be cooled on ice for 2 min. One micro-spoon of polyvinylpolypyrrolidone will be added to each tube, followed by vortexing and centrifugation for 3 min at 18,000 x g. The supernatant will be removed and placed in a new 2 mL tube and the pellet will be washed with TENP and centrifuged for 3 min at 18,000 x g, and the new supernatant will be added to that which was harvested previously. The pooled tube will be centrifuged for 1 min at 18,000 x g and the supernatant will be transferred to a new 2 mL tube. One volume of isopropanol will be added to the supernatant, gently mixed by turning the tube and incubated for 10 min at RT. After centrifugation for 5 min at 18,000 x g, the pellet will be resuspended in 0.1 M phosphate buffer, pH 8, and 5 M potassium acetate and incubated overnight at 4°C. The samples will then be centrifuged for 30 min at 18,000 x g and 4°C. The supernatant will be transferred to a new 2 mL tube, and after the addition of RNase (final concentration 40 µg/mL), incubated at 37°C for 30 min. Nucleic acids will be precipitated with absolute ethanol and 3 M sodium acetate, followed by centrifugation at maximum speed for 3 min. The pellet will be washed with 70% ethanol, dried and resuspended in 100 µL of TE buffer. DNA quality and concentration will be estimated by agarose gel electrophoresis and nanodrop (NanoDrop ND-1000) measurement, respectively.

Considering the low biomass samples, background controls have been made throughout the sampling and extraction process. The physiological serum used to perform BAL is first sampled with the same syringe that is afterward used for lavage from the same vessel containing physiological serum. This sample is used as a “negative sampling control”. During the DNA extraction, miliQ water is used as a “negative background control” sample, treated with all the

reagents and passing all the procedures along with the real samples. These “negative” samples will be analysed along with the real samples.

All the reagents used in DNA extraction and sample pre-treatments were either autoclaved, filtered through 20µm filters or purchased sterile. All the tools and pipettes were thoroughly washed and disinfected between extractions of different sample types, to minimise the transfer from high biomass samples. Also, DNA extraction from lung tissue samples (three samples per patient) was randomised (each extraction “batch” never contained only one sample type from different patients or all the samples from the same patient), to minimise the “batch” effect.

2.7.7 Molecular analyses of microbiota

16S rRNA gene sequencing. The genomic DNA from saliva, faeces, BALF, “healthy” lung, peritumoural and tumoural tissue, and negative controls will be analysed by sequencing of the bacterial 16S rRNA gene by DNA Vision, Belgium, using Illumina MiSeq technology. After PCR amplification of the targeted region V3-V4, libraries will be indexed using the NEXTERA XT Index kit V2. The sequencing is carried out in paired-end sequencing (2 x 250 bp) by targeting an average of 10,000 reads per sample. Software used for bioinformatic analysis will be QIIME (Quantitative Insights Into Microbial Ecology), with a cut-off value of 5,000 reads per sample for analysis. For each sample the following will be determined:

- a) alpha and beta diversity,
- b) comparison of alpha diversities based on a two-sample t-test using non-parametric (Monte Carlo) method,
- c) statistical significance of sample groupings using distance matrices (Adonis method),
- d) comparison of OTU frequencies across sample groups (comparison is performed at OTU, Phylum, Class, Order, Family and Genus level)

Multiple comparisons will be realised, both between different grouping criteria and between samples.

Considering the low taxonomic levels (Species), sequencing is best used as an indicative tool for further analyses by qPCR.

qPCR. In our study, qPCR will be done in two phases:

a) Pre-16S sequencing analysis

In this phase, qPCR will have two purposes: (i) to confirm and further characterise the bacterial functional groups in faeces evaluated by bacterial culture (providing the information of viable bacteria inside specific functional group); and (ii) to quantify pathogens/commensals in respiratory and intestinal system, known to be implicated in tumorigenesis/pro or anti-inflammatory reactions²⁷⁻³² using specific primers.

qPCR will provide information of absolute quantity of taxa/species of interest in each sample, which is information that cannot be obtained by sequencing (only relative abundance).

b) Post-16S analysis

In our study, the sequencing has the purpose of sample “screening”. It will give us an idea of the composition of the microbial communities from different sites and originating from different conditions (patient with tumours eligible for chemotherapy/surgery) based on minimum of 5,000 reads. However, sequencing stays a technique to determine relative abundance. Therefore, once the overall composition of each sample is determined, we will proceed with:

1. quantification of the specific outer taxonomic units (OTUs)/taxa we determine as relevant in either relative or normalised abundance not analysed during pre-16S analyses
2. enlarging the primer list specific for the new discovered OTUs of interest

The current list of primers optimised for our study is shown in Table 2. Each primer couple is tested for specificity on 60 referent species. QPCR will be done using the Rotor-Gene Q machine (Qiagen). Additional primer couples will be tested and optimised if found necessary, as explained.

Table 2 The current list of qPCR primer couples

Target group	Primer sequence (5' – 3')	Reference
Total bacteria	F CGGTGAATACGTTCCCGG	Furet <i>et al.</i> , 2009 ³³
	R TACGGCTACCTTGTTACGACTT	
<i>Bacteroidetes</i>	F AACGCTAGCTACAGGCTTAACA	Dick & Field, 2004 ³⁴
	R ACGCTACTTGGCTGGTTCA	
<i>Actinobacteria</i>	F TACGGCCGCAAGGCTA	Trompette <i>et al.</i> , 2014 ⁶
	R TCRTCCCCACCTTCCTCCG	
<i>Firmicutes</i>	F GGAGYATGTGGTTTAATTCGAAGCA	Guo <i>et al.</i> , 2008 ³⁵
	R AGCTGACGACAACCATGCAC	
<i>Gammaproteobacteria</i>	F CMATGCCGCGTGTGTGAA	Mühling <i>et al.</i> , 2008 ³⁶
	R ACTCCCCAGGCGGTCDACTTA	
<i>Bacteroides/Prevotella</i> (<i>Bacteroidales</i>)	F CCTWCGATGGATAGGGGTT	Layton <i>et al.</i> , 2006 ³⁷
	R CACGCTACTTGGCTGGTTCAG	
<i>Lactobacillus/Leuconostoc/</i> <i>Pediococcus</i>	F CGCCACTGGTGTTCYTCCATATA	Furet <i>et al.</i> , 2009 ³³
	R AGCAGTAGGGAATCTTCCA	
<i>Blautia</i> genus	F GTGAAGGAAGAAGTATCTCGG	Kurakawa <i>et al.</i> , 2015 ³⁸
	R TTGGTAAGGTTCTTCGCGTT	
<i>Veillonella</i> genus	F GRAGAGCGATGGAAGCTT	Tana <i>et al.</i> , 2010 ³⁹
	R CCGTGGCTTTCTATTCC	
<i>Neisseria</i> genus	F CTGTTGGGCARCWTGAYTGC	Yan <i>et al.</i> , 2015 ⁴⁰
	R GATCGGTTTTRTGAGATTGG	
<i>Fusobacterium</i> genus	F AAGCGCGTCTAGGTGGTTATGT	Dalwai <i>et al.</i> , 2007 ⁴¹
	R TGTAGTTCGCTTACCTCTCCAG	
<i>Bacteroides thetaiotaomicron</i>	F GACCGCATGGTCTTGTTATT	Haugland <i>et al.</i> , 2010 ⁴²
	R CGTAGGAGTTTGGACCGTGT	
<i>Bilophila wadsworthia</i>	F CGTGTGAATAATGCGAGGG	McOrist <i>et al.</i> , 2001 ⁴³
	R TCTCCGGTACTCAAGCGTG	
<i>Akkermansia muciniphila</i>	F CAGCACGTGAAGGTGGGGAC	Collado <i>et al.</i> , 2007 ⁴⁴
	R CCTTGCGGTTGGCTTCAGAT	
<i>Escherichia coli</i>	F CATGCCGCGTGTATGAAGAA	Huijsdens <i>et al.</i> , 2002 ⁴⁵
	R CGGGTAACGTCAATGAGCAAA	
<i>Faecalibacterium prausnitzii</i>	F GGAGGAAGAAGGTCTTCGG	Ramirez-Farias <i>et al.</i> , 2008 ⁴⁶
	R AATCCGCCTACCTCTGCACT	
<i>Blautia (Ruminococcus) gnavus</i>	F GGACTGCATTTGGAAGTGTGAG	Le Leu <i>et al.</i> , 2015 ⁴⁷

	R	AACGTCAGTCATCGTCCAGAAAG	
<i>Ruminococcus torques</i>	F	GCTTAGATTCTTCGGATGAAGAGGA	Le Leu <i>et al.</i> , 2015 ⁴⁷
	R	AGTTTTTACCCCCGCACCA	
<i>Bifidobacterium bifidum</i>	F	CCACATGATCGCATGTGATTG	Malinen <i>et al.</i> , 2005 ⁴⁸
	R	CCGAAGGCTTGCTCCCAA	
<i>Enterococcus hirae</i>	F	GGCATATTTATCCAGCACTAG	Daillère <i>et al.</i> , 2016 ⁴⁹
	R	TAGCGTACGAAAAGGCATCC	
<i>Pseudomonas aeruginosa</i>	F	CCAGCCATGCCGCGTGTGTGA	Silva-Junior <i>et al.</i> , 2016 ⁵⁰
	R	GTTGGTAACGTCAAAACAGCAAGG	
<i>Streptococcus pneumonia</i>	F	ACGCAATCTAGCAGATGAAGCA	Chien <i>et al.</i> , 2013 ⁵¹
	R	TCGTGCG TTTAATTCCAGCT	
<i>Haemophilus influenza</i>	F	AGCGGCTTGTAGTTCCTCTAACA	Fukumoto <i>et al.</i> , 2015 ⁵²
	R	CAACAGAGTATCCGCCAAAAGTT	
<i>Fusobacterium nucleatum</i>	F	CAAGCGGTGGAGCATGTG	Fukumoto <i>et al.</i> , 2015 ⁵²
	R	CTAAGATGTCAAACGCTGGTAAGG	
<i>Moraxella catarrhalis</i>	F	GGTGAGTGCCGCTTTTACAAC	Fukumoto <i>et al.</i> , 2015 ⁵²
	R	TGTATCGCCTGCCAAGACAA	
<i>Klebsiella pneumoniae</i>	F	CGGGCGTAGCGCGTAA	Fukumoto <i>et al.</i> , 2015 ⁵²
	R	GATACCCGCATTCACATTAACAG	

2.7.8 Statistical analysis plan

General information. This study is exploratory and main outcomes address the description of microbiota characteristics in lung cancer patients. Three microbiota are concerned (gut, lung and saliva) and data are collected at different times in patients treated by neoadjuvant chemotherapy (group Pct-chir). For other patients (group Pchir), different microbiota are sampled at only one time point, at surgery.

In patients treated by chemotherapy, analysis of variations of microbiota induced by chemotherapy will be possible by evaluation of changes in proportions over time. On the other hand, the analysis of microbiota with/without previous chemotherapy will only be descriptive, as the design is not compatible with a non-biased comparison of both groups.

Sample size. The sample size calculation is based on the study of Montassier *et al.*, 2015,⁵³ where the abundance of the principal phylum *Firmicutes* in faecal microbiota decreased by approximately 30%, with an FDR-corrected p-value of 0.0002. Considering these results, 20

patients in the chemotherapy group should allow us to detect a similar variation in our samples, at least in the faecal microbiota. For group balance, the same sample size of 20 was retained for the surgery-only group.

Description of patients' characteristics. Patients' characteristics will be described using standard distribution parameters: counts, range, mean/median, confidence intervals, standard deviation/interquartile range for quantitative parameters and, for categorical ones, counts and frequencies. This description will also be made by treatment group (Pchir/Pct-chir).

Description of microbiota. Microbiota characteristics consist in several hierarchical steps including:

- **Phylum:** four main phyla are found in both lung and intestinal microbiota (*Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*). *Firmicutes* are supposed to represent ~80% of the intestinal microbiota biomass⁵⁴ and ~40% of the lung microbiota.⁵⁵ The proportion of each component will be described by its proportion of the biomass in %.
- **Main classes of bacteria per phylum:** these classes gather bacteria that share important characteristics and functions (e.g. *Bacilli*, *Clostridia*, *Gammaproteobacteria*, etc.). Composition of the microbial communities from different sites and originating from different conditions will be quantified (alpha and beta diversity, relative proportions).
- **Inside classes,** description by order, family and genus level will be performed when contributory on a biological plan.

Heterogeneity between microbiota will be studied. The comparison of proportions of phyla, or by other taxonomic level will be performed to evaluate if specific adaptation characterises the three microbiota and their components. ANOVA will be used to perform inter-patient comparisons. FDR correction will be applied when analyses are conducted within phyla.

Comparison of microbiota before/after chemotherapy. This comparison will be performed for each site-specific microbiota. Proportions of main phyla will be compared using ANOVA (mixed model) to check if an independent chemo-effect can be objectivised, adjusting on patients and phyla (without FDR correction).

Comparisons of taxonomic levels below phylum before/after chemotherapy will be performed on relevant components with an FDR correction. These comparisons will be performed on both alpha and beta diversity. Univariate paired parametric or non-parametric tests (Student t-test, Mann-Whitney U-test, etc.) will be used here.

Comparison of microbiota between the two treatment groups. These comparisons will use the same tests as in the previous paragraph, except tests will not be paired.

Relationship between inflammatory status and microbiota. Several cytokines will be measured in blood samples. Each result will consist in a concentration of cytokine. The relationship between microbiota and inflammation will be tested using Pearson (or Spearman rank) correlation coefficient, using FDR correction.

Tumoural immune infiltration. Immune reaction will be described by percentage by lymphocyte type. These proportions will be compared to corresponding microbiota characteristics: e.g. lung microbiota and lung tumour. Statistical association between these parameters will be tested as in the previous paragraph.

Complementary analyses. Complementary analyses will be performed if particular biological issues can be better described.

All statistical analyses will be performed using R-software version 3.5.0 or later (R-Project, GNU GPL). Tests will be two-sided and the significance threshold is set at 0.05, after FDR correction where needed (as for the analyses concerning taxonomic ranks below phylum). Data may be missing due to possible loss of follow-up between inclusion and end of study. A description of the missing data and associated reasons will be given.

2.7.9 Data monitoring committee

A data monitoring committee is not needed in this study since this is an observational trial and there are no intervention or security risks for patients.

3 Discussion

What is known. Lung cancer is a leading cause of death by cancer worldwide, responsible for 1,761,007 or 23.1% deaths in 2018 according to the WHO.⁵⁶ It is also the most frequent cancer in men and the third most frequent in women.⁵⁶ While well characterised regarding its aetiology, morphological and molecular properties,⁵⁷⁻⁵⁹ much less is known regarding its relationship with lung microbiota, and almost nothing regarding its connection to distant sites such as the gut and gut microbiota. This lack of studies is self-explanatory when one knows that not so long ago lungs were considered sterile except in case of infection.^{55,60} Recently, however, there is an emerging idea of more “systemic” influence of the gut microbiota, and its connection to the immune system beyond the local effect.^{3,60-62} A few teams made a huge leap in elucidating the

role of the gut microbiota in chemotherapy and anticancer treatment, including lung cancer.^{63–67}

What is new. Based on these studies and the questions unanswered, we designed a case-control observational trial underlining a multi-aspect approach to the patient. In each of our subjects, we decided to characterise the microbiota of different sites (UAs, gut, and lung microbiota), in parallel with immune profile characterisation (local and systemic) while taking into account the patient's life style (nutrition, smoking status, profession, etc.). Examination of these factors in patients undergoing chemotherapy before surgery enables a direct follow up of these parameters correlated with the treatment phase (to our knowledge, this has never been reported for lung cancer before). Moreover, lung microbiota at surgery is sampled in two ways: by performing broncho-alveolar lavage (BAL) directly on the excised lung lobe (to eliminate possible UAs contamination and obtain the maximal microbial concentration for analysis), and by sampling lung tissue at 3 sites: “healthy” tissue distal to tumour (used as a control tissue),¹² peritumoural tissue, and tumour itself. This enables sampling of both luminal and tissue/cell-bound bacteria which, according to known studies, do not share the same microbial composition.²⁶ To our knowledge, at present there is no study of lung cancer that examines the microbiota of peritumoural tissue, and even less in 4 different lung sample types. Also, no study performed BAL directly on the tumour lobe without passing through the UAs.

Choice of analyses. As previously reported, certain bacterial species can modify our immune responses differently, such as *Faecalibacterium prausnitzii* or on the other hand *Fusobacterium nucleatum*, as well as the whole cluster (*Clostridia* cluster XIV).^{68,69} Therefore, tumour lymphocyte infiltration and lymphocyte composition in the broncho-alveolar lavage fluid (BALF) will be examined and closely looked at for its relationship with sampled microbiota. Likewise, each tumour is characterised according to the TNM stage classification⁷⁰ and histological properties. Tumour architecture, localisation in the lung, and disease severity are expected to dynamically interact with microbial composition *in situ* and immune profile, as seen in similar pathologic states of the lung (obstruction of the normal lung architecture, creation of anaerobic thermal pockets in the case of bronchial obstructiveness, immunogenicity of the tumour, promotion of neutrophil recruitment, inflammation).^{12,15,71–73} Difference of the microbiota composition between tumour samples of adenocarcinoma and squamous cell carcinoma has already been evidenced by Yu *et al.*¹⁵ Interestingly, salivary microbiota is also proven to correlate with NSCLC type.⁷⁴ Therefore, similar analysis direction will be taken with our salivary and lung samples.

As mentioned, intestinal microbiota has both local and systemic influence on its host. According to the gut-lung axis theory,⁴ bacteria or their products might have systemic effects, and therefore, have an effect on the lung microbial composition and immune response. For this reason, faecal microbiota will be characterised, as well as faecal SCFAs concentrations (known products of bacterial fermentation and immune modulators/protectors of the intestinal barrier).⁷⁵ SCFAs might be potential mediators of the gut's "long-distance" influence by direct effect on the target site or indirectly via gut/circulating immune system stimulation. As intestinal microbiota is shown to adapt very quickly to the changes in nutrition, as well as its influence on SCFAs concentrations,^{22,76} nutritional records before each faecal sampling will be taken into account. We will not only determine the composition of faecal microbiota, but also its "quality" and influence on intestinal health by dosage of bacteriocins (HNP1-3, β -defensin 2), and calprotectin as inflammatory marker.⁷⁵ Broad-spectrum cytokine profiling and immune cell phenotyping in the blood will be used to evaluate systemic immune status. This holds particular importance as connection to circulating IL-6 and IL-8 was previously reported in lung cancer,^{77,78} but also to intestinal SCFA concentrations.⁷⁹

As explained, group Pct-chir will enable follow-up on multi-site microbiota and immune status during different treatment phases (Figure 1). Since the biggest problem of chemotherapy, despite its efficacy against tumour cells, is its non-selectivity (effecting epithelial layers and mucosae),⁸⁰⁻⁸² we expect to see changes in all three types of microbiota – salivary, faecal and lung, as all are closely related to epithelial and mucosal layers. Similarly, immune characteristics should be altered following the chemotherapy and above-mentioned changes in microbiota (but also *vice versa* – the affected immune system will change its interaction with microbiota, thus modifying it). Finally, we could hypothesise that different initial properties of the tumour (why the patient is prescribed chemotherapy or not in the first place) might divide two patient profiles (Pchir vs. Pct-chir 1st time point) regarding both multi-site microbial and immune/inflammatory characteristics.

Final word. To conclude, our results will be one of the first to give a better understanding of the close and intense interaction between the microbiota of different, yet communicating sites and their interaction with the immune system in patients suffering from lung cancer (the world's number one cause of death by cancer).⁸³ The strength of this study design is data collection through multiple non-invasive techniques that can be incorporated into the standard medical care and treatment of the patients. Another strong point is a multi-site approach towards each patient: lifestyle, nutrition, immune status, and microbial composition are assessed using

different and complementary techniques (e.g. faecal microbiota will be assessed by techniques of molecular biology via qPCR and sequencing, but also by bacterial culture, and in the aspect of individual's nutrition). The main limitation is lack of the "healthy" control group, not authorised by Ethics Committee because of the invasiveness of the sampling techniques for healthy subjects. Therefore, previously published data on healthy subjects and similar cohorts will be addressed only in a descriptive matter, while we will focus more on relational aspects (e.g. interaction between site-specific immunity and its microbiota).

We hope that our results will help in setting the basis for developing more personalised or "alternative" approaches in lung cancer treatment, better characterisation of patient's status and diagnosis, as well as in finding ways of improving chemotherapy tolerance and effectiveness (complementary prebiotics, probiotics or symbiotics).

4 Authors' contributions

RB and EF wrote the manuscript. MF and EF proposed the study design and protocol preparation. JYB and ET were involved in the preparation of protocol amendments. MPV was involved in the study design. ABD and NRR led the study design and protocol preparation. FK wrote the statistical analysis plan. MF was responsible for patient recruitment, surgery procedure and lung sampling during surgery. Preparing the study design, sample and data collection, management, analyses and decision to submit the report for publication is the responsibility of RB, EF and MF. All authors have reviewed this manuscript.

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6 Competing interests

The authors declare that they have no competing interests.

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Results

Article 1: Characterisation of the four lung microbiota

Research Article

Characterisation of microbiota in saliva, bronchoalveolar lavage fluid, non-malignant, peritumoural and tumour tissue in non-small cell lung cancer patients: cross-sectional clinical trial

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Even though studies on lung cancer microbiota started to emerge several years ago, the characteristics of the lung microbiota originating from different sample types are still not well documented. The major problem of lung microbiota research is the fact that sampling methods and target samples are not uniform across the studies, meaning that the majority of the microbiota analysis is based on BAL, followed by tissue, saliva and sputum in varying frequency. Even though it was previously suggested that the lung microbiota from BAL and lung tissues harbours different communities, so far no study explicitly considered this subject. Moreover, analysis of the microbiota in lung cancer implies tissues with distinct roles, interactions and immunity, such as peritumoural and tumour tissue.

Therefore, in this study we provide detailed characteristics of the microbiota from four different lung samples: BAL, non-malignant, peritumoural tissue and tumour, with the addition of saliva as the sample of oral microbiota. We show that BAL indeed represents a unique microbiota based on differential abundance analysis and on beta diversity. Simultaneously, we confirm that BAL form a clear cluster of lung microbiota with the other lung tissues samples when put in the perspective of the oral microbiota. Next, since lobe position was suggested as important factor influencing the microbiota composition (adapted island hypothesis), and in the same time, lower lobe tumours have been associated to worse prognosis in NSCLC, we examined the influence of the tumour position in upper or lower lobes on analysed microbiota. We show that all analysed microbiota, except the one from the tumour, have significantly different abundance especially in the phyla *Bacteroidetes* and *Actinobacteria*. Moreover, we show that peritumoural tissue microbiota is the most sensitive to lobe location, with significantly increased resemblance to BAL microbiota when found in upper lobes. Since tumour is known for its potential to

directly modify its microenvironment, we propose that this observed change in peritumoural tissue microbiota actually reflects the changes in microenvironment (adhesive sites, extracellular matrix rearranging, immune stimulation). Finally, we show that phylum *Firmicutes*, previously reported as elevated in progressed lung malignancies, is elevated in abundance in all lung tissues from lower lobes when compared to upper lobes. This might be a potential microbial indicator of increased aggressiveness of lower lobe tumours, but will need further confirmation and analysis of its association with the immune system.

Research Article

Characterisation of microbiota in saliva, bronchoalveolar lavage fluid, non-malignant, peritumoural and tumour tissue in non-small cell lung cancer patients: cross-sectional clinical trial

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Abstract

Background. While well characterised on its molecular base, non-small cell lung cancer (NSCLC) and its interaction with local microbiota remain scarcely explored. Moreover, current studies vary in source of lung microbiota, from bronchoalveolar lavage fluid (BAL) to tissue, introducing potentially differing results. Therefore, the objective of this study was to provide detailed characterisation of the oral and multi-source lung microbiota of direct interest in lung cancer research. Since lung tumours in lower lobes (LL) have been associated with decreased survival, characteristics of the microbiota in upper (UL) and lower tumour lobes have also been examined.

Methods. Using 16S rRNA gene sequencing technology, we analysed microbiota in saliva, BAL (obtained directly on excised lobe), non-malignant, peritumoural and tumour tissue from 18 NSCLC patients eligible to surgical treatment. We provided detailed taxonomy, diversity and core analysis for each microbiota, with analysis of differential abundance on all taxonomical levels (zero-inflated binomial general linear model with Benjamini-Hochberg correction) between samples and lobe location.

Results. Diversity and differential abundance analysis showed clear separation of oral and lung microbiota, but more important, of BAL and lung tissue microbiota. Phylum *Proteobacteria* dominated tissue samples, while *Firmicutes* was more abundant in BAL and saliva (with class *Clostridia* and *Bacilli*, respectively). However, stratification between lobes showed increased abundance of *Firmicutes* in LL lung microbiota, with decrease in *Proteobacteria*. Also, clades *Actinobacteria* and *Flavobacteriia* showed inverse abundance between BAL and extratumoural tissues depending on the lobe location. While tumour microbiota seemed the least affected by location, peritumoural tissue showed the highest susceptibility with markedly increased similarity to BAL microbiota in UL. Differences between the three lung tissues were however very limited.

Conclusions. Our results confirm that BAL harbours unique lung microbiota and emphasize the importance of the sample choice for lung microbiota analysis. Further, limited differences between the tissues indicate that different local tumour-related factors, as tumour type, stage or associated immunity, might be the ones responsible for microbiota-shaping effect. Finally, the “shift” towards *Firmicutes* in LL might be a sign of increased pathogenicity, as suggested in similar malignancies, and connected to worse prognosis of the LL tumours.

Trial registration. ClinicalTrials.gov ID: NCT03068663. Registered February 27, 2017. <https://clinicaltrials.gov/ct2/show/NCT03068663>

1 Introduction

Despite the advancements in its detection and treatment, lung cancer (LC) is still the leading cause of death by cancer worldwide [1]. Non-small cell lung cancer (NSCLC) is diagnosed in 85-90% of LC cases and presents the most frequent type of lung cancer. Unlike small cell lung cancer, NSCLC is operable in 20-25% of cases. This concerns mostly early stage tumours (stage I and II), sometimes locally advanced disease (stage III) and rarely oligometastatic disease (stage IV). Other treatments, such as chemotherapy, radiotherapy and until recently immunotherapy, are often associated with surgery as neoadjuvant or postoperative treatment. Even though surgery is recognised as the most effective initial treatment of NSCLC, the 5-year survival rates remain however low (~90% for stage IA1, and ~12% for stage IIIC)[2,3]. Therefore, the tumour staging is used as the important prognostic tool based on tumour size, lymph node invasion and metastatic status [2]. Curiously, tumour lobe location has also been associated to tumour's aggressiveness, with tumours in lower lobes (LL) showing worse term and 5-year survival after resection than the ones in upper lobes (UL), still without a clear explanation [4–6].

Increasing interest in the interaction between host and its microbiota revealed its potential implication in health and disease, but also in tumour immunology and physiology [7–11]. Unlike local and systemic effects of the gut microbiota, the lung microbiota and its effects remain scarcely explored, being only recently accepted as the one of the resident microbiota (and not only present during infection)[12,13]. Since, emerging number of studies turned to its exploration, notably in the context of cystic fibrosis, asthma and chronic obstructive pulmonary disease (COPD), interstitial lung disease, and lung transplantation [14–19]. Despite its impact on global cancer-related death, lung cancer studies were left surprisingly few-numbered and started to emerge only a few years back. However, they confirmed that lung microbiota interacts with local immunity and modifies tumour properties. The microbial dysbiosis in antibiotic-treated or germ-free animals influenced growth of injected lung tumour cells [20,21] while usage of penicillin, cephalosporins, or macrolides showed to increase risk of lung cancer in human subjects [22]. In lung cancer patients, lung microbiota from bronchoalveolar lavage fluid (BAL) enriched with supraglottic taxa was associated with pro-inflammatory profile and stimulation of Th17 cells with protumourigenic effect [23–25], and also exhibited different

abundance and metabolic profiles compared to the one of healthy subjects [26,27]. Interestingly, salivary microbiota was also found to show cancer specific profile, with genera *Veillonella* and *Capnocytophaga* more abundant in saliva of lung cancer patients [28]. At the present, only two studies analysed lung tissue microbiota in lung cancer. One found increased alpha diversity in non-malignant tissue compared to tumours as well as in adenocarcinoma compared to squamous cell carcinoma [29], while the other showed association between increased diversity of the non-malignant tissue (but not tumour) and decreased recurrence-free and disease-free survival [30]. Among studies on lung microbiota, those on BAL are the most numerous, since it remains the sample with acceptable ratio of contamination risk by upper airways, precision in lung microbiota sampling and invasiveness. However, this has been a potential source of contradictory information since varying characteristics of BAL and tissue microbiota, as a result of samples' different nature, have been previously suggested [18]. Therefore, there has been an increasing necessity to characterise the ground differences between different lung microbiota in NSCLC patients to enable better comprehension of the obtained results depending on the initial lung sample.

With the primary objective to fill this “missing link”, this cross-sectional pilot study analysed lung microbiota from four different samples in 18 NSCLC patients eligible for surgery without neoadjuvant therapy. The analysed samples were BAL, non-malignant tissue, peritumoural tissue and tumour, as each should represent different architectural and physiological characteristics. Unlike in previous studies, BAL was obtained by direct sampling from the excised lobe without passing through the upper airways, to decrease aforementioned risk of contamination. In addition, salivary microbiota was characterised for each patient and used as an extra-pulmonary sample (to put in perspective the relation with and between lung samples). As a second objective, we investigated whether tumour location in the UL or LL yields significant changes in these microbiota.

2 Methods and patients

2.1 Patient recruitment and study design

All patients were enrolled in a prospective, study, approved by the CPP Sud Est VI Ethics Committee and registered at ClinicalTrials.gov (NCT03068663) [31]. Written informed consent was obtained from all patients before enrolment in the study and any study procedure.

Patients diagnosed with primary NSCLC eligible for surgical treatment with or without neoadjuvant therapy and presented before the Thoracic Oncologic Committee of the Jean Perrin Centre (Clermont-Ferrand, France) were preconsidered for inclusion to the study. Inclusion criteria were: age between 18 and 80 years, body mass index (BMI) < 29.9, no antibiotics, corticoids, immunosuppressive drugs or underwent pulmonary infections for at least the past 2 months, as well as no previous airway surgery or cancer treatment. Only patients included into the group of patients eligible for surgery without chemotherapy were taken into account in this manuscript.

At inclusion, patients received the tube for saliva collection and were asked to bring it with them the day of their hospital admission for surgery. Sampling of the lung was performed during the surgery immediately after excision of the tumour lobe, representing no additional inconvenience for patient apart its standard medical procedure. Detailed inclusion/exclusion criteria, the study flowchart as well as detailed design and power calculation were previously published [31].

2.2 Sampling

2.2.1 Saliva

Any evidence of oral health problems or injuries were recorded for each patient at inclusion. Each patient received a tube for saliva collection (Sarstedt) that he or she filled with minimum of 1 mL of saliva (designated on the tube) on an empty stomach by the passive drooling method on the morning of hospital admission for the surgery. Patients were asked to keep the sample stored on cold. Upon reception by the study personnel, the sample was stored at -80°C until DNA extraction.

2.2.2 Lung tissue and BAL

Sampling of lung tissue and BAL during surgery was performed immediately after partial or complete pneumonectomy. The removed lung or lung lobe was placed in a sterile vessel and the tumour position was determined by palpation. First, a piece of non-malignant lung distal to the tumour (opposite side of the lobe) with an average size of 1 cm³ was clamped. The clamp was left in place until the end of the following procedure. Using a sterile syringe the excised lung was inflated through the main bronchus. Bronchoalveolar lavage was performed by instilling 2 x 40 mL of sterile physiological saline into the bronchus. After each instillation, the maximum amount of liquid inside the bronchus was retrieved (8-10 mL in total) into 50 mL tube (designated as "BAL"). Then, the clamped wedge of non-malignant tissue was cut off and

designated as LUNG.DP (“distal piece”). Further, a pie-slice of the tumour (cross-section) was excised with its peritumoral tissue, after which the two were separated based on macroscopic histological difference. Tumour tissue sample was designated as “LUNG.T” and peritumoral tissue sample as “LUNG.PT”. The tissues were snap-frozen in liquid nitrogen and then placed at -80°C for long-term storage until DNA extraction. BAL was stored directly at -80°C .

2.3 DNA extraction and negative controls

DNA was extracted as previously described [31]. Saliva and BAL (cellular BAL) volume used for DNA extraction were 1 and 5 mL, respectively. Initial tissue weights ranged from 377 ± 236 mg for LUNG.PT, to 1.441 ± 1.016 g and 1.346 ± 0.899 g for LUNG.DP and LUNG.T. Even though the initial weight between tissue samples was significantly different ($p = 0.001$), there was no difference in final concentration of DNA/g of sample ($p = 0.895$). DNA extraction from lung tissue samples (three samples per patient) was randomised (each extraction group never contained only one sample type or all samples from the same patient) to randomise the manipulation effect.

Since the lung samples are considered as a low biomass samples, background controls were made throughout the sampling and extraction procedure. Negative sampling control was collected for each BAL and consisted of physiological serum collected with syringe used for instillation from the same liquid recipient. During the DNA extraction, milliQ water was used as a negative background control and underwent same procedure as the real samples. All controls were sequenced and analysed. Reagents used in DNA extraction and sample pre-treatments were either autoclaved, filtered through $20\ \mu\text{m}$ filters or purchased sterile. All tools and pipettes were thoroughly washed and disinfected before and after each extraction cycle or between different extraction steps.

2.4 16 ribosomal RNA (16S rRNA) gene sequencing

DNAVision (Belgium), using Illumina MiSeq technology, performed 16S ribosomal rRNA gene sequencing. Following the PCR amplification of the targeted region V3-V4, libraries were indexed using the NEXTERA XT Index kit V2. The sequencing was carried out in paired-end sequencing (2 x 250 bp) by targeting an average of 10,000 reads per sample. Next, sample sequences were clustered into OTUs based on 97% sequence similarity. This was performed with software QIIME (Quantitative Insights Into Microbial Ecology). Further microbiota analyses were done on generated “raw” OTU table, taxonomy and Newick formatted phylogenetic tree provided by DNAVision.

2.5 Sequence processing and microbiota analysis

Microbiota analysis and visualisation were done with RStudio 3.5.2. [32] (packages “phyloseq” [33], “vegan” [34], “microbiome” [35], “ggplot2” [36], “DESeq2” [37], “metacoder” [38]).

Raw OTU table was filtered to keep only kingdom *Bacteria* for further analysis. The total of 26 negative controls (sampling and background) had in average 21 detected OTU with average of 4 reads/OTU and did not belong to more abundant OTUs in samples. These OTU counts were subtracted from corresponding samples, i.e. negative sampling control (physiological serum for washing) from corresponding BAL sample, and negative extraction controls from the samples in the extraction group. Samples were processed in two batches with controlled randomisation of all sample types and patients, including clinical data, so that both batches were equally diverse. Therefore, only OTUs present in all samples of one batch and not present in all samples of the other were excluded from further processing as a consequence of unequal extraction efficiency (and not of contamination). These preprocessing did not alter any of the measures, and have left the data virtually unchanged. Next, OTU present in at least 10 % of the lung samples and 20% of saliva samples or having more than 50 overall counts (for each group) were kept for further processing (in our case, this was equivalent of keeping the OTUs with minimal average abundance of 0.001% in either of groups). Only samples with more than 1000 reads were included in analysis. This excluded 6 samples (without preference for certain factor): 2 BAL, 3 peritumoural tissues (LUNG.PT) and 1 tumour (LUNG.T). Average read number of final sample groups was $39,083 \pm 9,697$ (mean \pm SD) for saliva, $17,046 \pm 14,879$ for BAL, $13,352 \pm 12,909$ for LUNG.DP, $13,039 \pm 11,394$ for LUNG.PT and $5,846 \pm 4,505$ for LUNG.T. For analysis of alpha and beta diversity, samples were rarefied at 1195 reads with 100 iterations. Observed OTU number, Shannon diversity index and Faith’s phylogenetic diversity were used for alpha diversity characterisation (“phyloseq”). Groups’ beta diversity was calculated based on weighted (importance of abundance and quality) and unweighted (importance of absence or presence of OTUs) UniFrac distances using function `adonis` (“vegan”) with 999 permutations and presented with non-metric multidimensional scaling (NMDS). Core microbiota clustering was based on Bray-Curtis distance and NMDS method.

Taxonomic trees (“metacoder”) representing relative abundance of taxa within each sample group were based on arithmetic mean of relative abundance calculated from unrarefied OTU table for each group. Input for trees representing differential abundance was calculated by DESeq function using zero-inflated method of negative binomial general linear model

(significant coefficient difference calculated by Wald's test) and Benjamini-Hochberg (BH) correction for multiple comparison with 0.05 threshold ("DEseq"). The model was used on unrarefied taxon counts.

Difference in alpha diversity and paired UF distances was calculated by Kruskal-Wallis or Mann-Whitney U test with BH correction for multiple comparison with 0.05 threshold of significance.

2.6 Managing missing data – paired/unpaired tests

Total of six lung samples were excluded from the study due to insufficient number of reads (<1000). Excluded samples did not originate from only one specific tissue, only one patient or exclusively belonged to one criteria. Therefore, preservation of strictly paired analysis would exclude an important number of other related samples. For this reason, analysis were unpaired if not specified otherwise.

3 Results

3.1 Participant characteristics

The total of 18 patients eligible to surgical treatment without neoadjuvant therapy was included in the study. Microbiota was analysed in 17 saliva and 68 lung samples, where 16 patients provided all 5 different samples (1 saliva plus 4 lung). For the analysis relative to the location of the tumour lobe, patients were grouped into two groups: 1st group with tumour in upper lobes (UL), and 2nd group with tumour in middle and lower lobes (LL) (share same descending bronchus). Patients with LL tumour had significantly lower predicted diffusing capacity of the lung for carbon monoxide (DLCO) than patients with tumours in UL ($p = 0.034$). Other clinical parameters showed no significant difference. Patients' characteristics (total and per lobe location group) and final sample number used in analysis after exclusion of samples with less than 1000 reads are shown in Table 1.

Table 1 Characteristics of patients included in the study

	Total	Upper lobe T	Lower lobe T	<i>p</i>
Total no. of patients	18	10	8	
Male/female	13/5	8/2	5/3	
Age (years)	68 ± 8	65 ± 9	72 ± 6	0.061
BMI	25 ± 3	25 ± 4	25 ± 3	0.859
T in upper/middle/lower lobe	10/2/6	10/0/0	0/2/6	
ADC/SCC/carcinoid	11/5/2	6/2/2	5/3/0	
Stage I/II/III	8/2/8	5/2/3	3/0/5	
Tumour size (cm)	3.7 ± 2.3	3.6 ± 2.4	3.9 ± 2.4	1
Smoker/ex-smoker/never-smoker	2/14/2	0/9/1	2/5/1	
Pack-year (smokers, ex-smokers)	31 ± 20	31 ± 21	33 ± 20	0.823
FEV ₁ (% of expected value)	98 ± 11	95 ± 8	101 ± 14	0.408
DLCO (% of expected value)	74 ± 16	81 ± 13	64 ± 16	0.034
FEV ₁ /FVC (% of expected value)	96 ± 10	95 ± 11	98 ± 10	0.630
Final no. of samples (statistics)				
Saliva	17	10	7	
BAL	15	8	7	
LUNG.DP	17	10	7	
LUNG.PT	14	9	5	
LUNG.T	16	9	7	

ADC – adenocarcinoma; BAL – bronchoalveolar lavage fluid, BMI – body mass index; DLCO - diffusing capacity of the lung for carbon monoxide; FEV₁ – forced expiratory volume per second; FVC – forced vital capacity; LUNG.DP – non-malignant tissue, LUNG.PT – peritumoural tissue, LUNG.T – tumour, SCC – squamous cell carcinoma; T – tumour

3.2 Beta diversity identifies BAL as a unique sample

Microbiota was analysed in saliva, bronchoalveolar lavage fluid (BAL), non-malignant Distal Piece (LUNG.DP), Peritumoural Tissue (LUNG.PT) and Tumour (LUNG.T). As expected, saliva showed a clear separation from the four lung samples (BAL and tissues) with significant difference in beta diversity based on both weighted (wUF) and unweighted (uwUF) UniFrac distances (Fig. 1A). Furthermore, lung samples showed significant separation based on both wUF and uwUF. The three lung tissues were all significantly different from BAL, with peritumoural tissue showing the least significant dissimilarity (wUF) compared to other two (Fig. 1A). There was, however, no significant difference between tissues. Looking at samples' position in NMDS (Fig. 1A), it was visible that BAL creates a clear cluster with lung tissues vs. salivary microbiota, but that from lung samples it is the one closest to saliva.

The four lung samples shared similar average values of observed OTU number (~120) as well as phylogenetic (~15) and Shannon diversity indexes (~3.5) (Fig. 1B, C, D). Although, compared to saliva, lung microbiota showed higher variance, all lung samples showed significantly higher phylogenetic diversity and higher number of observed OTUs (latter significant only for tumour) compared to salivary microbiota. However, in Shannon diversity saliva and lung samples were found around the same level (~3.5) (Fig. 1D).

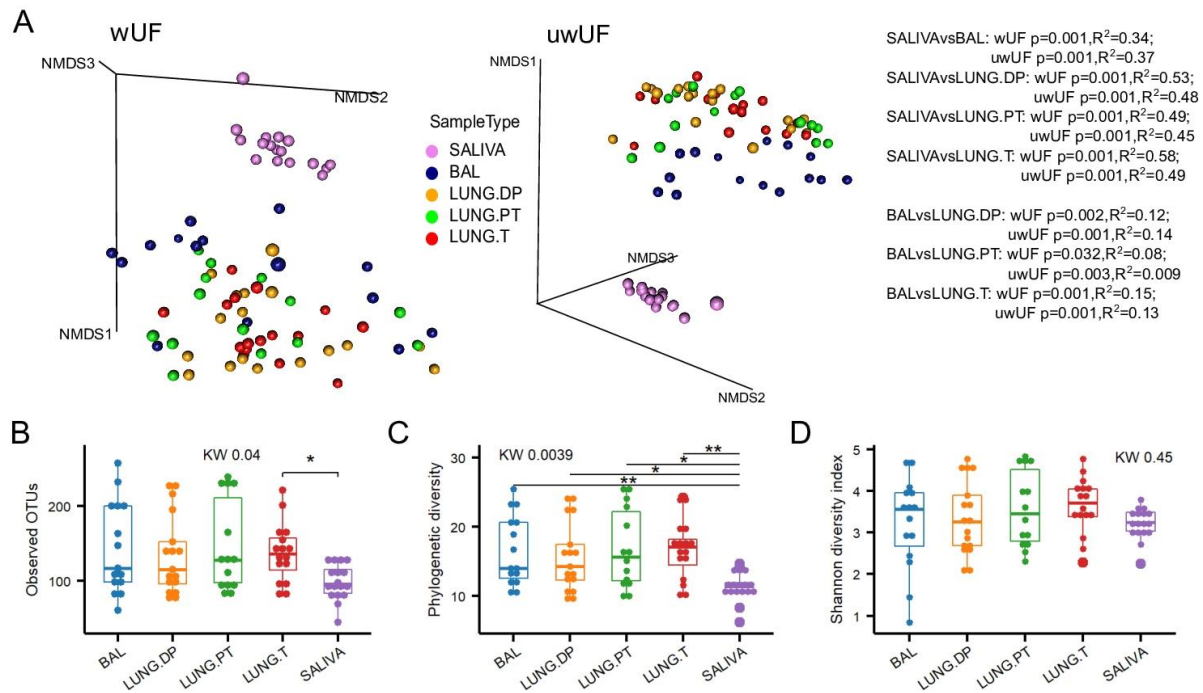


Figure 1 Diversity of the salivary and four lung microbiota. **a** Beta diversity of salivary and lung microbiota represented by non-metric multidimensional scaling (NMDS) based on weighted (wUF) and unweighted (uwUF) UniFrac distances. Alpha diversity of saliva and four lung samples assessed by **b** number of observed OTUs, **c** Faith's phylogenetic diversity, and **d** Shannon diversity. Statistical significance of difference in beta diversity was assessed with adonis function (vegan) with 999 permutations. Statistical significance of difference in alpha diversity was assessed with Kruskal-Wallis (KW) followed by, where appropriate, Man-Whitney U test with BH correction for multiple comparison. *: $p \leq 0.05$, **: $p \leq 0.01$. BAL - bronchoalveolar lavage fluid, LUNG.DP - non-malignant distal piece, LUNG.PT - peritumoural tissue, LUNG.T - tumour.

3.3 *Proteobacteria* and *Firmicutes* (class *Clostridia*) dominate lung samples

13 phyla, 29 classes (27 in BAL), 87 families (85 in BAL and tumour), and from 112 to 115 genera were detected in each of the lung samples. In saliva on the contrary, there were 10 phyla, 17 classes, 26 orders, 49 families and 68 genera. Composition of each sample is shown in Figure 2A in a form of a taxonomic tree with indicated average relative abundance of taxa next to taxon's name ($> 0.001\%$) and the number of samples in which they were detected (numbers within branches). The most abundant phyla and genera in each sample are synthetically presented in Figure 2B and 2C, respectively. The saliva tree was the least complex from all the trees, heavily dominated by the phylum *Firmicutes* (53.7%) and belonging genus *Streptococcus* (32.7%) (Fig. 2A,C). Other phyla as *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria* followed with decreasing abundance (Fig. 2B). Except *Streptococcus*, additional

11 genera had abundance higher than 1%, including *Prevotella*, *Veillonella*, *Neisseria*, *Porphyromonas*, and *Actinomyces* as the top five.

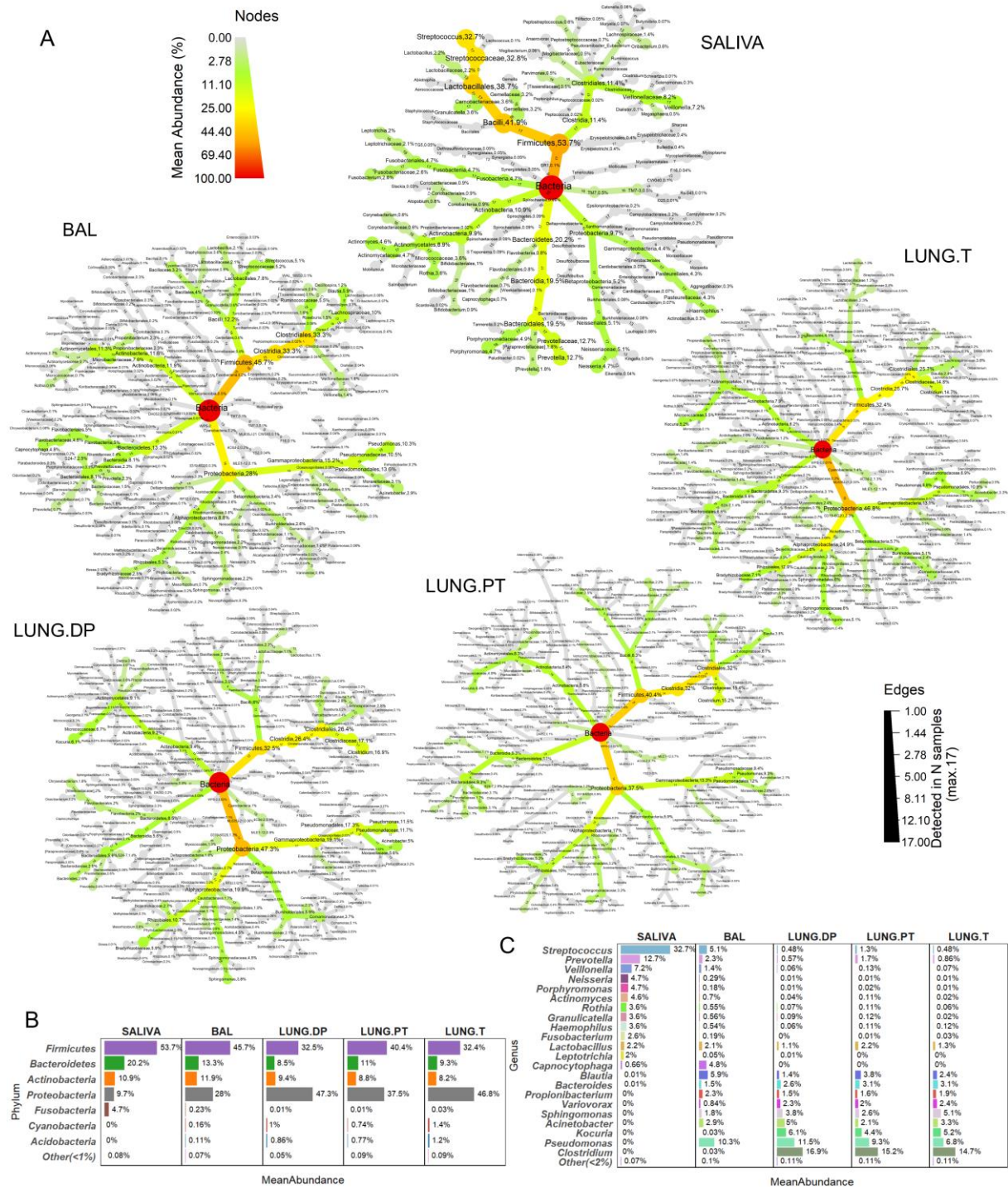


Figure 2 Relative abundance and prevalence of the four lung and salivary microbiota. a Each tree represents the taxonomical composition of one sample type. The colour and node size correspond to taxon abundance. All taxa with abundance higher than 0.001% are shown, and percentage is noted for all taxa with abundance higher than 0.01%. Number of samples within which the taxon was detected is noted within branches. Maximal number of samples is 17 for saliva and non-malignant tissue, 16 for tumour, 15 for BAL, and 14 for peritumoural tissue

(Table 1). Synthetic presentation of the most abundant taxa was provided on **b** phylum and **c** genus level. BAL - bronchoalveolar lavage fluid, LUNG.DP - non-malignant distal piece, LUNG.PT - peritumoural tissue, LUNG.T - tumour.

In the lung samples, two dominating phyla were *Proteobacteria* and *Firmicutes*, but with varying abundance ratio depending on the sample. So, phylum *Firmicutes* was the most abundant in BAL, in peritumoural tissue two phyla were equally abundant, while *Proteobacteria* dominated non-malignant tissue and tumour (Fig. 2A,B). Phyla *Bacteroidetes* and *Actinobacteria* were found in lower abundance in all lung samples (~10% each). Interestingly, while high abundance of phylum *Firmicutes* in saliva was almost entirely due to members of the class *Bacilli*, in lung samples it is due to class *Clostridia*, introducing one of fundamental differences between these two microbiota. Moreover, *Clostridia* was the most abundant class in all lung samples except the tumour, where it shared the highest abundance with the class *Alphaproteobacteria*. Compared to saliva, the whole phylum *Proteobacteria* was more developed in lung samples, containing additional large class of *Alphaproteobacteria*, but lacking *Epsilonproteobacteria* (detected in saliva).

On the genus level, there was no extensive prevalence by one genus as seen in saliva, but rather a group of representatives with different taxonomic origin (Fig. 2A, C). In the three lung tissues, *Pseudomonas*, *Clostridium*, *Kocuria*, *Acinetobacter* and *Sphingomonas* were the five most abundant genera, but in BAL, those were *Pseudomonas*, *Blautia*, *Streptococcus*, *Capnocytophaga* and *Acinetobacter* (Fig. 2C). Interestingly, two highly abundant genera in tissues, *Clostridium* (~15%) and *Kocuria* (~5%) were found in very low abundance in BAL. Inverse was seen for *Capnocytophaga*, that seemed to be a BAL-related genus (~5%), with a very low presence in saliva and absence in tissue samples. Furtherly, BAL was the only lung sample that had higher abundance of so-called supraglottic taxa, as *Streptococcus*, *Prevotella* and *Veillonella*, compared to other tissue samples. The abundance was also slightly higher in peritumoural tissue, supporting potentially increased similarity between BAL and peritumoural tissue seen in beta diversity (Fig. 1A).

3.4 Whole phyla and classes significantly different between lung samples and saliva, but also between BAL and tissues

Figure 3A shows difference in taxa abundance between samples. Taxa with significant difference are coloured, with a colour scale representing log₂ fold change in abundance between compared sample pair.

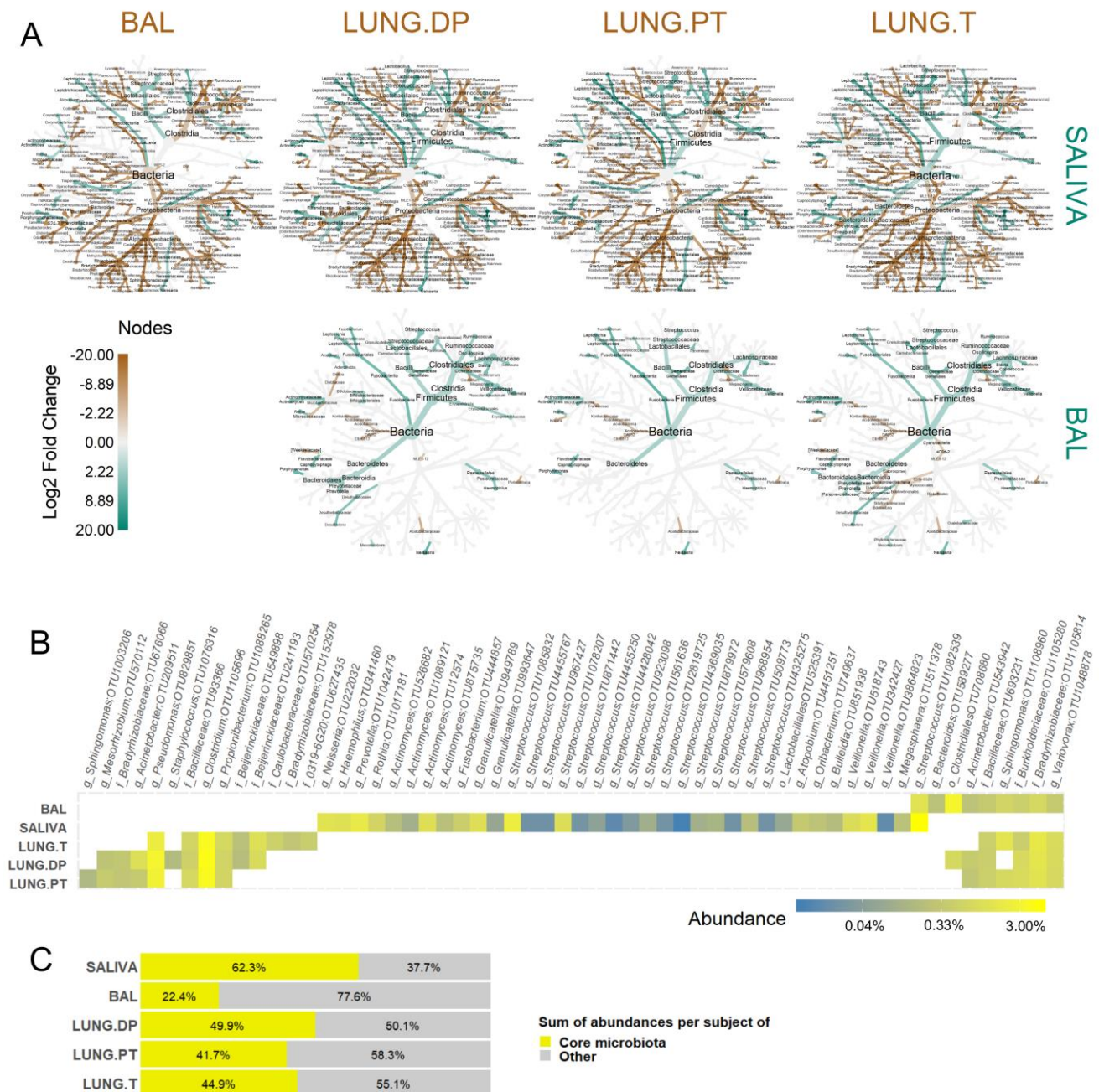


Figure 3 Differential abundance between lung and salivary microbiota and their core composition. **a** Coloured nodes and branches in each tree represent the taxa with significantly different abundance between two compared microbiota. The colour intensity is proportional to log₂-fold change in abundance in the favour of the sample with the same colour. Taxa names are shown in the common legend tree below comparisons. Statistical significance was assessed by zero-inflated general linear model using Wald’s test (DESeq), with p-value threshold of $\alpha \leq 0.05$ after BH correction. **b** Core microbiota determined as OTUs present in 100% of each sample for one sample type. The colour represents relative abundance on transformed log₄ scale. **c** Average value of sum of abundances of the core OTUs and of other OTUs per each subject in each sample types.

The first row (Fig. 3A) shows taxa with significantly different abundance between saliva and each of the four lung samples. Compared to saliva, all lung samples had significantly higher abundance in whole classes of *Alphaproteobacteria*, *Deltaproteobacteria*, *Cytophagia*,

Sphingobacteriia, [*Saprospirae*], and *Acidimicrobiia*, but also in whole phyla such as *Cyanobacteria*, *Acidobacteria*, *Nitrospirae*, *Verrucomicrobia* and *Planctomycetes* (latter not seen for BAL). Similarly, phyla *Synergistetes*, *Spirochaetes*, *Fusobacteria* and *TM7* (not in BAL), classes *Epsilonproteobacteria* and *Erysipelotrichi* were significantly more abundant in saliva than in any of lung samples. On the other hand, multiple descending members of several higher taxa were not strictly more present in only one sample type. This particularly concerned members in the classes from the principal phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. Significantly more abundant in saliva samples were orders *Neisseriales* (*Betaproteobacteria*), *Pasteurellales* (*Gammaproteobacteria*, contains *Haemophilus*), *Lactobacillales* (*Bacilli*, contains *Streptococcus*) and families *Veillonellaceae* (*Clostridia*), *Flavobacteriaceae* (*Flavobacteriia*), *Prevotellaceae*, *Porphyromonadaceae* (both *Bacteroidia*), *Actinomycetaceae* and *Corynebacteriaceae* (both *Actinobacteria*). Conversely, from the same higher taxa, significantly more abundant in lung samples were orders *Burkholderiales* (*Betaproteobacteria*), *Pseudomonadales*, *Legionellales*, *Xanthomonadales*, *Enterobacteriales* (both *Gammaproteobacteria*), *Bacillales*, *Turicibacteraceae* (both *Bacilli*), and families *Ruminococcaceae*, *Lachnospiraceae* (both *Clostridia*), *Bacteroidaceae* (*Bacteroidia*), *Propionibacteriaceae*, *Dietziaceae* and *Bogorellaceae* (*Actinobacteria*).

Second row in Figure 3A shows significant difference in abundance between BAL and the three lung tissues. With a few pair-reserved exceptions, pattern was highly similar between comparisons. Also, it was visible that most of the differences indicated significantly increased abundance in BAL compared to tissues. In BAL, significantly higher abundance was seen in phylum *Fusobacteria*, classes *Clostridia* and *Bacilli* (genera *Streptococcus*, *Veillonella*, *Roseburia*, *Oribacterium*, *Phascolarctobacterium*, *Parvimonas*, and *Megasphaera*), orders *Pasteurellales* (*Haemophilus*) and *Desulfovibrionales*, families *Actynomicetaceae*, *Flavobacteriaceae* (*Capnocytophaga*), and genera *Atopobium*, *Porphyromonas*, *Neisseria*, and *Rothia*. Adversely, the three tissue microbiota had only a few taxa with significantly higher abundance compared to BAL. Those were the whole phylum *Acidobacteria*, families *Acetobacteraceae* and *Clostridiaceae* (genus *Clostridium*), and genus *Perlucidibaca*.

However, there were differences in taxa that could be observed only between certain BAL-tissue pairs. Interestingly, the highest number of individual differences was seen in comparison between BAL and tumour microbiota. Here, only genus *Coprococcus* was significantly more abundant in BAL, while genus *Kocuria*, orders *Bdellovibrionales*, *Myxococcales*, *Rickettsiales*, and class [*Saprospirae*] were all significantly more abundant in tumour. Considering non-

malignant tissue microbiota, only family *Dietziaceae* was more abundant, while orders *Bifidobacteriales* and *Erysipelotrichales* were significantly more abundant in BAL. Interestingly, tumour and non-malignant tissue had important number of similar differences in comparison to BAL. While BAL had higher abundance of genera *Blautia*, *Granulicatella*, *Ruminococcus*, *Oscillospira*, *Prevotella*, and *Mezorhizobium*, more abundant in both tumour and non-malignant tissue were phylum *Cyanobacteria* and family [*Weeksellaceae*]. On the contrary, peritumoural tissue did not share any of these differences with BAL as did the other two tissues. In individual differences, genus *Kocuria* was the only significantly more abundant in peritumoural tissue, and inversely, only *Staphylococcus* was more abundant in BAL.

There were, however, no significant differences between three tissues.

3.5 Core OTUs in lung samples mostly members of phylum *Proteobacteria*

Core microbiota was determined as OTUs detected in 100% of samples in each group (Fig. 3B). The highest number of core OTUs was observed in saliva, with the total of 36. Two-fold less was seen in non-malignant tissue (16), peritumoural tissue (14) and tumour (14), and four-fold less in BAL (9). 75% of core OTUs in saliva belonged to phylum *Firmicutes*, with as high as 17/20 OTUs from genus *Streptococcus*, while additional 15% was from the phylum *Actinobacteria* (especially genus *Actinomyces*). In lung samples, 70% of the core OTUs belonged to the phylum *Proteobacteria* (1/3 from class *Alphaproteobacteria*) and other 30% to *Firmicutes*. Core OTUs were mostly shared between different lung sample types, especially between tissues. OTUs corresponding to genus *Variovorax* and unclassified members of families *Bradyrhizobiaceae*, *Burkholderiaceae*, and *Bacillaceae* were detected in all four lung microbiota, while OTUs for genera *Pseudomonas*, *Clostridium* and *Propionibacterium* were only common in all lung tissue microbiota. Even though 30% of lung core OTUs belonged to *Firmicutes*, only one OTU corresponded to genus *Streptococcus* and was a part of BAL core microbiota. This was also the only core OTU shared between saliva and lung samples, i.e. only BAL, which were otherwise clearly distinct. Within core microbiota (Fig. 3B), certain OTUs were uniquely associated with species, such as OTUs for *Rothia mucilaginosa*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, *Prevotella melaninogenica*, *Variovorax paradoxus*, *Veillonella parvula* (OTU 518743) and *Veillonella dispar* (other two OTUs). In average (Fig. 3C), core microbiota represented 62% of relative abundance in saliva,

against only 22% seen in BAL. In the tissues, the core microbiota represented around half of the total abundance, ranging from 42% to 50% of abundance.

3.6 Microbiota in lower lobes with higher abundance of *Firmicutes* and the diversity of peritumoural tissue as the most influenced by location

Next, we examined whether there is a significant difference between lung microbiota associated to the tumour lobe relative to its location. Two groups have been considered: upper lobes (UL) vs. middle/lower lobes (LL). Interestingly, only peritumoural tissue microbiota showed significantly different beta diversity between two locations in both wUF and uwUF (Fig. 4A, B). Moreover, difference in beta diversity between UL peritumoural tissue and BAL was not significant, unlike the one in LL (in UL: wUF $p = 0.17$, uwUF $p = 0.073$, vs. in LL: wUF $p = 0.004$, uwUF $p = 0.002$). To confirm this observation, for comparison we selected exclusively distances between each two samples originating from the same patient (Fig. 4D, E), i.e. paired distances. Indeed, in UL peritumoural microbiota was significantly more similar to both saliva and BAL, manifested as shorter distances compared to ones in LL (Fig. 4D,E: “distance to SALIVA”, “distance to BAL”). We next looked at the paired distances between the three tissue samples (Fig. 4D,E: “distance to LUNG.DP”). The paired distance between non-malignant tissue and the other two tissues, respectively, was inverse depending on the lobe location. In UL, there was an increased similarity between non-malignant tissue and tumour, and in LL, between non-malignant and peritumoural tissue. However, paired distance between peritumoural tissue and tumour remained unchanged (Fig. 4D,E: “distance to LUNG.PT”), suggesting a potentially balanced change or exchange of the microbiota maintaining the distance (and the difference) on the same level. This was inverse to observed by beta diversity including the totality of distances between tissue samples (not only between paired samples), showing that peritumoural tissue and tumour are compositionally different in the UL, significant for both wUF and uwUF (Fig. 4C). In the LL however, the difference was not significant ($p = 0.077$, $R^2 = 0.173$). So, based on overall microbiota composition, peritumoural tissue microbiota was significantly more similar to BAL microbiota in UL, but to tumour microbiota in LL.

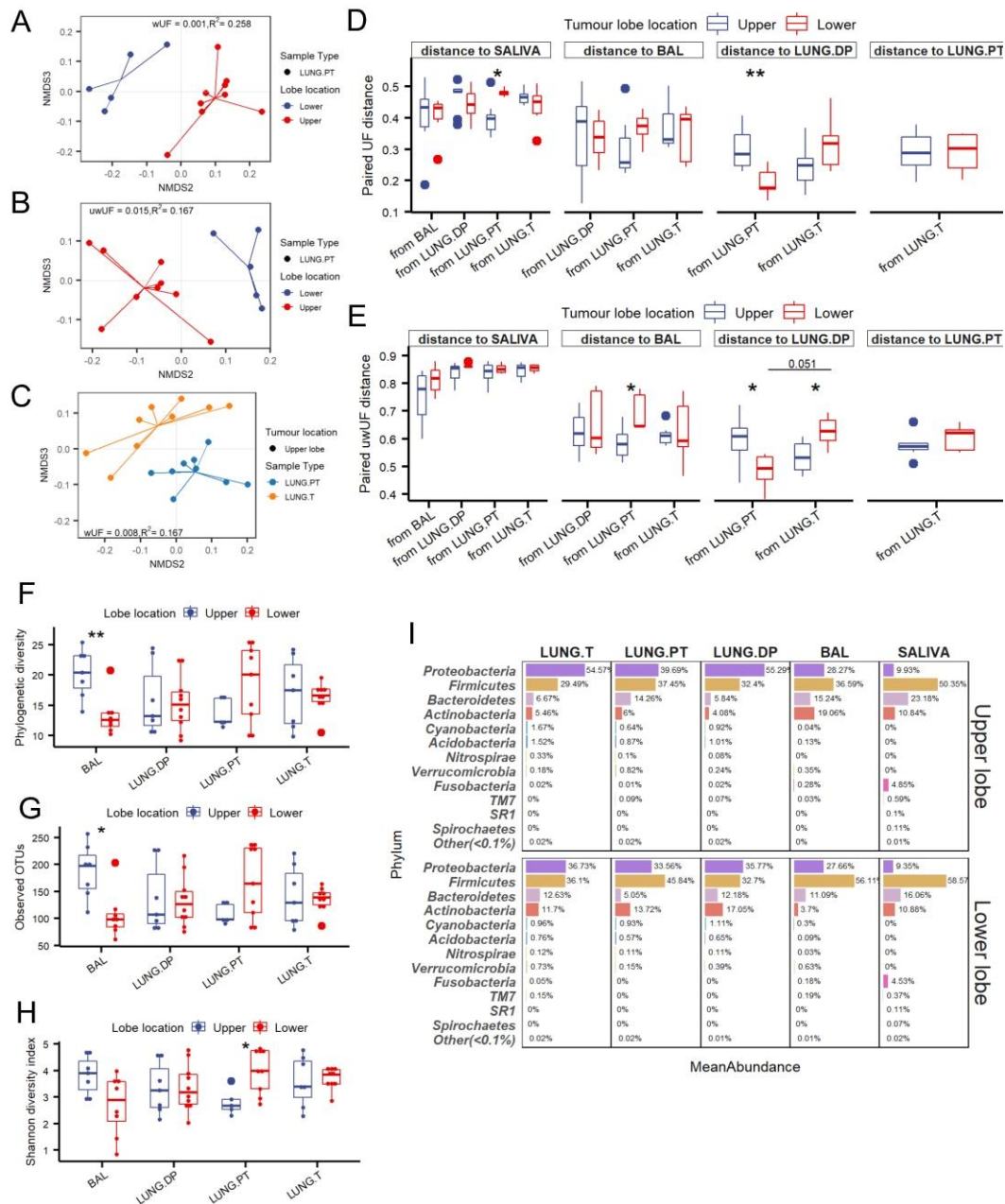


Figure 4 Diversity and predominant taxa in lung samples from upper and lower tumour lobes. Beta diversity found significantly different between peritumoural tissue from upper and lower lobes based on both **a** weighted (wUF) and **b** unweighted (uwUF) UniFrac distances. **c** Significantly different beta diversity based on wUF between peritumoural tissue and tumour in the upper lobe. **d** Weighted and **e** unweighted UF distances between samples coming from the same patient (i.e. paired distances) compared between upper and lower tumour lobes. The facet name represents the referent sample (e.g. “distance to BAL”) to which were calculated the distances noted on x-axis (e.g. “from LUNG.T”). Smaller distance indicates increased similarity. Alpha diversity for four lung samples between upper and lower tumour lobe assessed by **f** Faith’s phylogenetic diversity, **g** number of observed OTUs, and **h** Shannon diversity. **i** Most abundant phyla in each of the microbiota samples if the tumour is found in upper or lower lobes. Significance of difference in beta diversity was assessed with adonis function (vegan, 999 permutations). Statistical significance in alpha diversity and paired distances was assessed with Kruskal-Wallis followed by, where appropriate, Man-Whitney U test with BH correction

for multiple comparison. *: $p \leq 0.05$, **: $p \leq 0.01$. BAL - bronchoalveolar lavage fluid, LUNG.DP - non-malignant distal piece, LUNG.PT - peritumoural tissue, LUNG.T - tumour.

Both BAL and peritumoural tissue varied in alpha diversity depending on lobe location, unlike non-malignant tissue and tumour. BAL in LL had significantly lower phylogenetic diversity and number of observed OTUs (Fig. 4F,G) compared to UL BAL, and a tendency seen for Shannon diversity (Fig. 4H). Inversely, LL peritumoural tissue had significantly increased Shannon diversity (Fig. 4H), with tendency in phylogenetic diversity and number of OTUs.

In LL, there was a marked decrease in abundance of *Proteobacteria* and increase in phylum *Firmicutes* in each of the lung samples (Fig. 4I). Therefore, in LL tumour and non-malignant tissue had equal abundances in *Proteobacteria* and *Firmicutes* (~35%), while BAL and peritumoural tissue were both dominated by *Firmicutes* (56% and 45%, respectively).

3.7 *Actinobacteria* and *Flavobacteriia* show inverse abundance between BAL and extratumoural tissues depending on the lobe location, while tumour microbiota remains unchanged

Tumour location in UL or LL significantly influenced the microbial abundance in each of the analysed sample types, but not in the same manner (Fig. 5). As suggested by diversity results, microbiota of the peritumoural tissue seemed to be the most influenced by the lobe location. The changes were limited to members of three major phyla: *Firmicutes*, *Actinobacteria* and *Bacteroidetes*, and candidate phylum *TM7*. More precisely, in UL peritumoural tissue more abundant were class *Erysipelotrichi* and families *Gemellaceae*, *Streptococcaceae*, *Ruminococcaceae*, *Lachnospiraceae* and *Veillonellaceae* (all phylum *Firmicutes*), phylum *TM7*, classes *Bacteroidia* (phylum *Bacteroidetes*) and families *Actinomycetaceae* and *Bifidobacteriaceae* (phylum *Actinobacteria*). This abundance pattern was very similar to pattern of initial comparison between BAL and each of lung tissue samples (Fig. 2A). This could add to the results of beta diversity suggesting that UL peritumoural tissue was more similar to BAL than it was in LL. On the other hand, LL peritumoural tissue was enriched with three classes from phylum *Bacteroidetes* (*Flavobacteriia*, *Sphingobacteriia* and *Cytophagia*), and with families *Clostridiaceae* (genus *Clostridium*) and *Micrococcaceae* (genus *Kocuria*) from phylum *Firmicutes* and *Actinobacteria*, respectively.

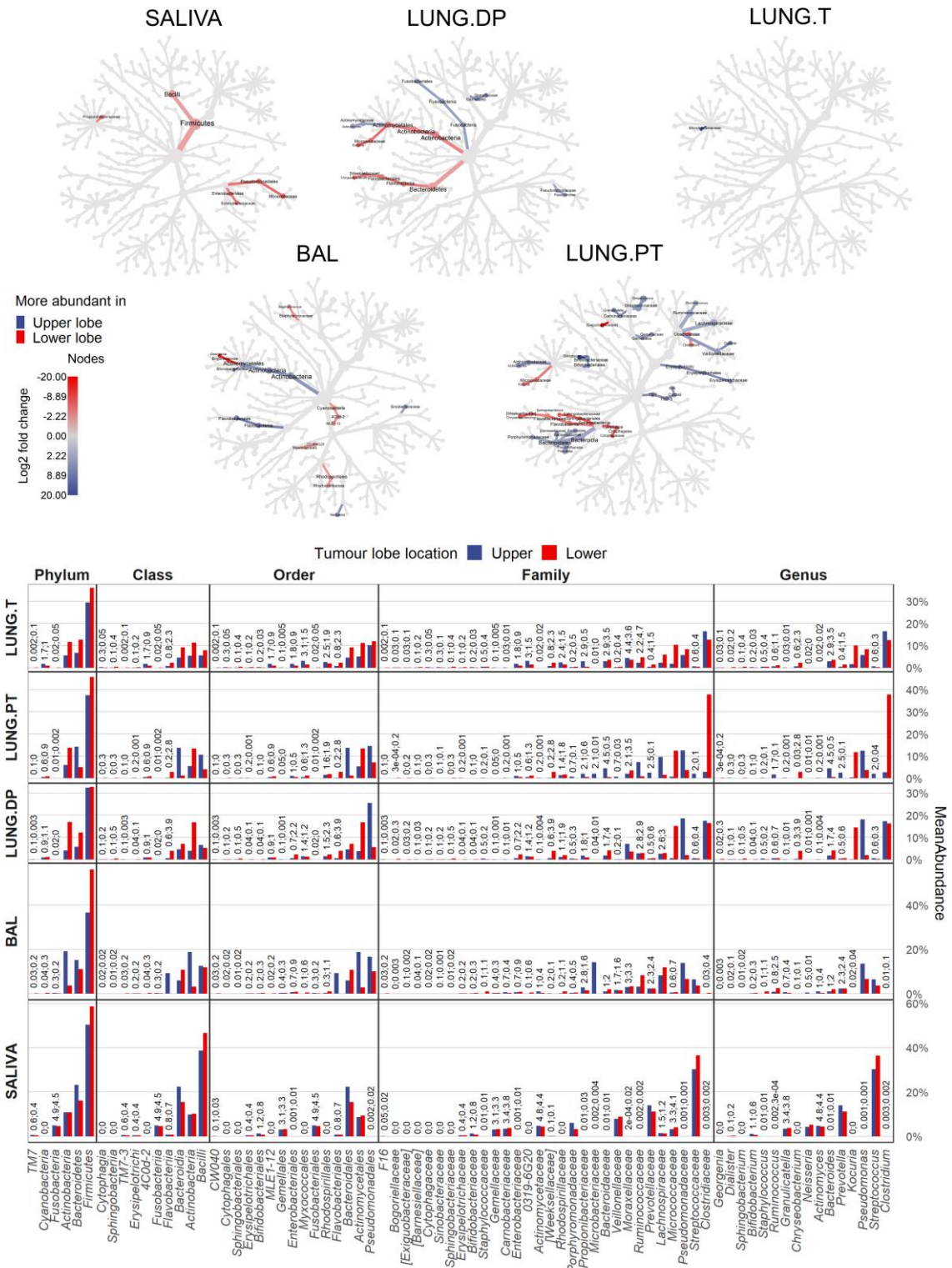


Figure 5 Differential abundance between upper and lower tumour lobes in salivary and lung microbiota. Each tree represents taxa with significantly different abundance relative to sample's origin (for lung) in the upper or lower tumour lobe. For saliva, the comparison shows the significant difference in salivary microbiota between patients with tumour either in upper or lower lobe. Coloured nodes and branches represent the taxa with significantly different abundance and the intensity is proportional to log2-fold change in abundance in the favour of the lobe noted with the same colour. Statistical significance was assessed by zero-inflated general linear model using Wald's test (DESeq),

with p-value threshold of $\alpha \leq 0.05$ after BH correction. Bar chart shows the relative abundance of taxa noted in the taxonomical trees. BAL - bronchoalveolar lavage fluid, LUNG.DP - non-malignant distal piece, LUNG.PT - peritumoural tissue, LUNG.T - tumour.

Compared to number of affected taxa in peritumoural tissue, both BAL and non-malignant tissue were less influenced by the lobe location, while tumour seemed to be almost entirely unaffected (Fig. 5). Moreover, phylum *Firmicutes* and class *Bacteroidia*, harbouring the most differences in peritumoural tissue, were not found significantly different in either non-malignant tissue or BAL. Instead, in UL non-malignant tissue was enriched with the phylum *Fusobacteria* and only a few other lower taxa without involvement of the whole clade (order *Gemellales*, families *Actinomycetaceae* and *Pseudomonadaceae*). Interestingly, in both non-malignant and peritumoural tissue phylum *Actinobacteria* (genus *Kocuria*) and class *Flavobacteriia* (genus *Chryseobacterium*) were significantly more abundant in LL, while in BAL these same two taxonomic groups were significantly more abundant in UL, opposite from the two issues. As seen from the relative abundance (Fig. 4I, Fig. 5), rather than moving from the common starting point, the abundances were inverse between samples (e.g. *Actinobacteria* in UL for BAL vs tissues was 19.06 vs ~5%, and in LL was 4% vs ~13%, respectively). Further, in UL BAL, genus *Neisseria* and family *Sinobacteraceae* (both *Proteobacteria*) were more abundant along with the mentioned two clades of *Actinobacteria* and *Flavobacteriia*. In LL BAL, more abundant taxa were more dispersed between taxonomic groups and included the whole phylum *Cyanobacteria*, several taxa from the phylum *Proteobacteria* (orders *Rhodospirillales*, *Myxococcales*), and two genera with their orders, *Staphylococcus* (phylum *Firmicutes*) and *Georgenia* (phylum *Actinobacteria*).

Even though importance was given to log₂ fold change of abundance, certain taxa held however high impact on the overall composition of the samples due to their higher relative abundance (Fig. 5, Additional file: Fig. 1). This was particularly true for three genera, *Clostridium*, *Kocuria* and *Pseudomonas*. In peritumoural tissue, genus *Clostridium* (phylum *Firmicutes*) ranged from 3% in UL vs 38% in LL, in non-malignant tissue genus *Pseudomonas* (*Gammaproteobacteria*) represented 18% in UL vs 2% in LL lobes, and genus *Kocuria* (phylum *Actinobacteria*) ranged from 0.5% and 0.2% in UL to 12% and 15% in LL in peritumoural and non-malignant tissue, respectively.

Finally, saliva samples, as the extrapulmonary sample with no direct physical connection to the tumour location as lung microbiota, also showed significantly different abundance profile relative to tumour lobe location. If tumours were found in LL, saliva was significantly enriched

in class *Bacilli* and families *Enterobacteriaceae*, *Moraxellaceae* (both *Gammaproteobacteria*) and *Propionibacteriaceae* (*Actinobacteria*). Curiously, no taxa were detected as significantly more abundant if tumour was found in UL.

3.8 Stratification between lobes defines differences between BAL and tissues and confirms similarity of BAL and peritumoural tissue in upper lobes

Stratification of samples by lobe location also revealed or defined differences between samples (Fig. 6). Between saliva and the four lung samples there was little change, since these samples were initially already very different (Fig. 3). Only in UL, there was no more difference in phylum *Nitrospira*, and orders *Neisseriales* and *Haemophilus* between BAL and saliva (difference significant for all other saliva vs lung comparisons in both lobes, including LL BAL). Also, the individual difference between lobes (Fig. 5, Fig. 4I) resulted in significantly higher abundance of *Actinobacteria* in UL BAL, but inversely, significantly lower abundance of *Actinobacteria* in LL BAL compared to saliva (Fig. 6). Comparing saliva and the three lung tissues, only taxa *Spirochaetes*, *Sphingobacteria* and *TM7* showed changes relative to the lobe location. Phylum *Spirochaetes* was significantly more abundant in saliva only when compared to UL non-malignant and UL peritumoural tissue (otherwise, no significant difference). Class *Sphingobacteria* was significantly more abundant in all lung tissues when compared to saliva, except in UL peritumoural tissue. This was interesting because neither UL nor LL BAL showed this difference, once again adding to similarity of BAL and peritumoural tissue in UL. Lastly, *TM7* was significantly more abundant in both UL and LL non-malignant tissue compared to saliva. But in UL, *TM7* was also more abundant in tumour, while in LL this was true for peritumoural tissue (other comparisons no significant difference). Despite these changes and stratification by location, saliva was still predominantly abundant in *Firmicutes*, *Fusobacteria*, *Epsilonproteobacteria*, *Erysipelotrichi* *Prevotella* and *Neisseria* clade, while lung samples dominated in abundance of *Proteobacteria*, *Acidobacteria*, *Nitrospirae*, *Verrucomicrobia*, *Cyanobacteria*, and the rest of *Bacteroidetes*.

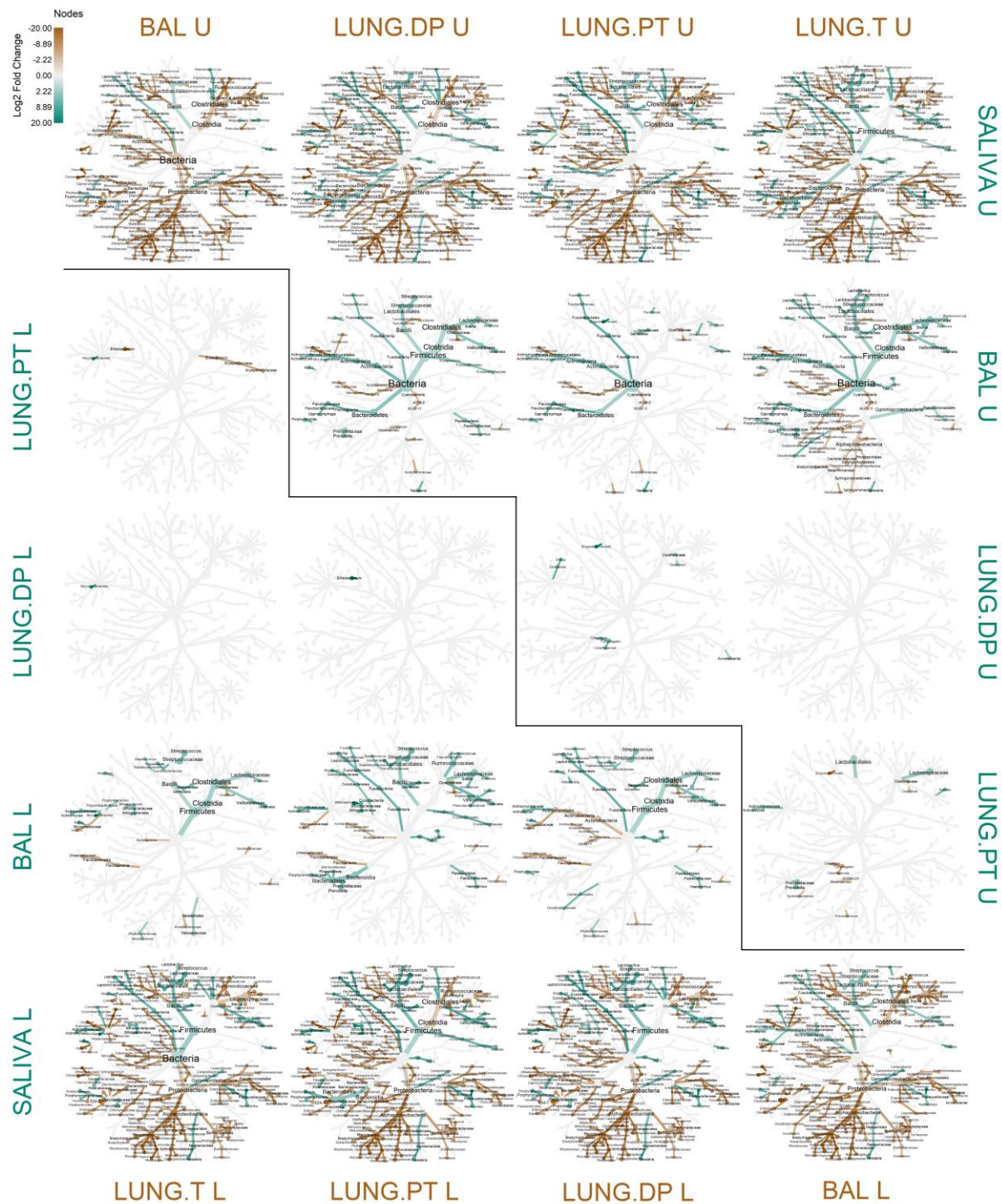


Figure 6 Comparison of abundance between salivary and lung microbiota relative to tumour lobe location. Two parts of the figure represent comparison in abundance between samples linked to upper (U) lobes in the upper part of the figure and to lower (L) lobes in the lower part of the figure. Coloured nodes and branches represent taxa with significantly different abundance between the two compared samples. The colour intensity is proportional to log₂-fold change in abundance in the favour of the sample with the same colour. Statistical significance was assessed by zero-inflated general linear model using Wald's test (DESeq), with p-value threshold of $\alpha \leq 0.05$ after BH correction. BAL - bronchoalveolar lavage fluid, L - lower lobe, LUNG.DP - non-malignant distal piece, LUNG.PT - peritumoural tissue, LUNG.T - tumour, U - upper lobe.

Unlike the comparison between saliva and lung samples, the aspect of lobe location induced much more developed and varying differences between BAL and tissues. Following the individual inverse difference observed in Fig. 5, in UL, *Flavobacteriia* and *Actinobacteria* were significantly more abundant in BAL, while in LL they were more abundant in the tissues. Also, the two phyla, *Nitrospirae* and *Cyanobacteria* (which between UL BAL and saliva showed no difference) were consequently more abundant in tissues only in UL, while there was no difference with BAL in LL. Interestingly, differences in phyla *TM7* and *Acidobacteria* came in pair. The significantly increased abundance of *TM7* in BAL was accompanied with increased abundances of *Acidobacteria* in the tissues, otherwise no difference was seen in either of them. In UL, this was seen in tumour, while in LL this was seen for peritumoural and non-malignant tissue. Observing the trees, it was visible that the highest number of differences was found between UL tumour and BAL, including large portion of detected taxa within *Proteobacteria*, especially *Alphaproteobacteria*, as more abundant in tumour. Even though other comparisons included a few members of *Proteobacteria*, the count was not as important as in UL tumour. Further, differences between UL BAL and extratumoural tissues were quite similar, except of the involvement of *Firmicutes* in peritumoural tissue. While in UL differences were scarce, in LL the pattern is as the one seen in comparison to other tissues. This is in accordance to Fig. 5, where significantly higher abundance of *Firmicutes* was detected in UL peritumoural tissue compared to LL. This could be, therefore, one of the major factors behind increased similarity between BAL and peritumoural tissue in UL. Detailed individual differences between BAL and tissues can be found in Additional file 1: “Additional explanation of differences between lung samples”.

Finally, several significant differences were noted between tissues (Fig. 6). They are, however, few numbered. Curiously, in both locations, more difference was found between tumour and peritumoural tissue, than between tumour and non-malignant tissue (none and one in UL and LL, respectively). Differences were also found between two extratumoural tissues. Interestingly, in each location, several detected differences between tissues addressed same taxa. Therefore, in UL, class *Cytophagia*, family *Exiguobacteraceae* and *Clostridiaceae* were the least abundant in peritumoural tissue compared to other two tissues, while in LL family *Microbacteriaceae* had significantly lowest abundance in tumour. In overall, differences were dispersed within four most abundant phyla and considered mostly endpoint taxa. Also, three tissues were more similar in LL than in UL. Detailed individual differences between the tissues

can be found in Additional file 1: “Additional explanation of differences between lung samples”.

4 Discussion

Following the last advancement on the interaction between gut microbiota, immune system and the tumour environment [8–11], there has been a growing interest in studying this concept in other physiological environments involving extraintestinal tumours. However, before exploring the effect of the gut microbiota, there has been an increasing necessity to investigate the effect of the local microbiota on the tumour as well [39]. Despite the emerging number of studies on the lung microbiota in different malignancies [14,40–43], its involvement in lung cancer is in its promising beginnings [23,27,29,30,44]. However, for the moment there is still no study considering the ground difference between different lung samples and their microbiota, while it is suggested that those could harbour microbiota with varying characteristics [18], and therefore, have diverging interactions with local immunity and tumour.

This study is, to our knowledge, the first to characterise the lung microbiota originating from four different lung samples (BAL, non-malignant tissue, peritumoural tissue and tumour), accompanied with the characterisation of salivary microbiota in NSCLC patients. We hypothesised that samples’ nature, “architecture”, physiological functions and environment, will influence characteristics of associated lung microbiota. Therefore, BAL should represent “planktonic” bacterial population found within the bronchial lumen or associated to biofilms or mucus [45], sampled along due the hydrodynamic force of the instilled liquid. Non-malignant tissue from the same lobe but taken on the opposite side from the tumour should represent a sample with a normal lung architecture, with well-defined small alveolar spaces and single-cell epithelial layer. In majority, it should harbour the “normal” biofilm, mucus and cell-associated lung microbiota. On the contrary, tumour represents a tissue with disrupted architecture, varying in form and obstruction degree relative to its type and grade [2]. Tissue modelling could also involve overproduction of the mucus as seen in certain subtypes of adenocarcinoma [46], but also different reaction of the immune system [47]. Finally, peritumoural tissue represents a non-malignant tissue in the direct contact with the tumour, separated as based on different histological properties. In literature it is addressed as tumour microenvironment and harbours different roles in stimulation or suppression of tumour metabolism [48]. Therefore, we presumed that its characteristics would be different both from the distal non-malignant tissue and the tumour. Except the number of different lung samples, the particularity of this study is

also in the way of obtaining BAL. It was obtained directly on the excised lobe containing tumour without using the bronchoscope, to minimise contamination risk of upper airways and increase the precision in characterisation of “true” BAL microbiota in the tumour proximity.

We reported that the four lung samples significantly clustered versus oral microbiota based on beta diversity, a confirmation of a previous result in healthy subjects that lung microbiota is distinct from other communities [29]. Salivary microbiota had several lower alpha diversity metrics compared to lung microbiota, as well as high dominance of genus *Streptococcus* and overall phylum *Firmicutes*. Along with its homogenous core microbiota (again prevalence of *Streptococcus* OTUs) that represented almost $\frac{3}{4}$ of total relative abundance per patient, this indicated its inter-subject stability and lower complexity compared to the one of the lung samples. In addition, the significant difference in abundance including the majority of the taxa detected in samples clearly placed the four lung samples versus oral microbiota. We identified typically oral taxonomic groups more abundant in saliva in all comparisons, such as *Fusobacteria*, *Spirochaetes*, *Synergistetes*, *Erysipelotrichi*, *Epsilonproteobacteria*, *Bacilli* and *Neisseriales*, most of them in concordance with previous literature [49,50]. On the other hand, phyla *Acidobacteria*, *Cyanobacteria*, *Nitrospira*, *Verrucomicrobia* and *Planctomycetes* were detected as strictly lung-associated (detected in each lung sample) with no representatives in saliva.

Next, we showed that BAL microbiota, even though undoubtedly belonging to lung microbiota cluster, had significantly different features from tissues. First, multidimensional representation of the beta diversity placed BAL samples on the side of the lung cluster towards salivary microbiota. Even though presence of more abundant salivary taxa was very limited in both tissue samples and BAL, *Streptococcus*, *Prevotella* and *Veillonella* (the three most abundant genera in saliva) were found elevated in BAL compared to tissue samples. It was previously suggested that these and other typically oral bacteria are found in low abundance in healthy lungs, due to their constant elimination [51]. Since BAL represents microbiota of the bronchial lumen, which undergoes constant influx of upper airways particles by respiration and is also the first “space” influenced by microaspiration [52], those could explain increased presence of these supraglottic taxa in BAL compared to the lung tissues. The same reasoning could explain the pattern of significant differences between samples. Indeed, taxonomic groups significantly more abundant in saliva when compared to lung microbiota highly corresponded to groups significantly more abundant in BAL when compared to lung tissues. Second, lung samples had *Proteobacteria* and *Firmicutes* as the two most abundant phyla, however with different ratios.

While BAL was dominated by *Firmicutes*, almost twice as abundant as *Proteobacteria*, lung tissues were dominated by *Proteobacteria* (as previously reported [29]), or at best, had equal abundance of the two (peritumoural tissue). Here it is however important to note that the high abundance of phylum *Firmicutes* in lung samples was due to members of the class *Clostridia*, unlike in saliva, where it was due to highly abundant class *Bacilli*. This emphasises one of the essential differences between oral and lung microbiota often omitted by selective presentation of only phylum or genus level. Third feature was that BAL did not differentiate from lung tissues only by difference in taxa abundance, but also by the presence of BAL-specific bacteria, such as genus *Capnocytophaga*, or absence of tissue specific-bacteria, such as genus *Kocuria* or *Clostridium*. The two latter genera were, however, detected in BAL, but with relative abundance 175-fold and 520-fold lower (~0.03%), respectively, than in the tissues. This detection is possibly due to their presence in host's cells during the DNA extraction, since cellular BAL was used for BAL analysis [45]. Lastly, no differences in alpha diversity metrics were detected between four lung samples, suggesting two different but equally "rich" lung microbiota populations. All this supports the hypothesis that BAL indeed represents a unique lung microbiota in lung cancer and that concerns of diverging results due to different samples have been justified [18].

Conversely to our hypothesis, lung tissues "as-they-are" did not show significant differences in abundance or diversity. Although increased diversity of non-malignant tissue versus tumour has been previously suggested [29], we did not find any difference. This could be due to the fact that our study group was more balanced in patients with less and more advanced tumour stages. In referenced study, the majority of subjects had tumours in stages I and II, manifesting decreased diversity compared to higher stages according to authors.

We also reported that the existence of the core OTUs and that these were mostly shared between lung tissues and partially with BAL, consisting in the majority of the members of *Proteobacteria*. Interestingly, one of the core OTUs was uniquely associated to species *Variovorax paradoxus*, ambiguous aerobic organism found in many niches, also in oral microbiome, and known for its potent biofilm creation [53,54]. It was detected as the core OTU in all four lung samples, while conversely, it was not found in saliva (where it was originally detected). This could suggest its potential role in biofilm creation in lower airways and would be an interest topic for future verification and investigation of potential role in pathogenesis of lung cancer [55].

Even though still under debate, several studies connected lower lobe tumours with worse prognostics [5,56,57]. Therefore, we examined the characteristics of the lung, but also salivary microbiota, if the tumour was found in upper or lower lobes. Indeed, we noted several changes in alpha and beta diversity but they were focused on changes in BAL and peritumoural tissue (with lower and higher alpha diversity, respectively, in LL compared to UL). We reported that in all four lung samples, lower lobes had decreased abundance of phylum *Proteobacteria* into favour of *Firmicutes*. Increased abundance of phylum *Firmicutes* was previously seen in BAL of LC patients compared to patients with benign-mass lesions [44], but also in patients with COPD [18]. In our study, 62% of patients in the LL were diagnosed with Stage III tumours, against 30% in the UL. Even though this stays a small study, this finding supports that “shift” from *Proteobacteria* to *Firmicutes* might have a role in lung cancer progression.

We showed that location significantly influenced the abundance of two clades, *Actinobacteria* and *Flavobacteriia*, in the inverse manner between BAL and extratumoural tissues. While both are more abundant in UL BAL, their abundance in UL extratumoural tissues was 2-10 fold lower, with exact inverse situation in LL. Both genus *Chryseobacterium* sp. (*Flavobacteriia*) and *Kocuria rhizophila* (*Actinobacteria*) detected as more abundant in LL tissues have been previously reported as uncommon human pathogens [58,59]. However, their increased presence selectively in LL tissues and their important overall abundance (~4% and 12%, respectively) suggest that there might be an important communication between different lung environments depending on local conditions or malignancy status, influencing preferential bacterial growth in one type of considered environment.

Interestingly, we found that salivary microbiota was also susceptible to the tumour lobe location. All differences considered taxa elevated in case of LL tumours and included families previously associated with bacterial exacerbations and infections (*Moraxellaceae*, members of *Bacilli* as *Streptococcus*, *Staphylococcus*, etc.) [60] or pulmonary complications (*Propionibacteriaceae*, *Enterococcaceae*)[61–63]. However, these taxa were not found elevated in LL lung microbiota. This, however, does not exclude their implication in malignant changes or being its consequence, and will be interesting topic for further consideration.

Finally, we found that peritumoural tissue showed higher similarity to BAL in UL both in beta diversity and in abundance, while in LL it shared characteristics with other tissue samples. Moreover, we found that alpha diversity of peritumoural tissue increased in LL to the level of other tissues, while the ones of BAL decreased. Peritumoural tissue is the tissue in the direct contact and interaction with the tumour. In the history of cancer research, the role of

extracellular matrix (ECM) surrounding tumour has been extensively studied [64]. Tumour cells are found to be able to directly influence the rearranging of connective units (such as collagen) and degradation of ECM to create tumour-permissive environment and enabling metastatic progression (Altinay 2016) [65,66]. In our case, it is possible that the observed changes in peritumoural tissue composition reflect certain remodelling of ECM, which could therefore be either more or less permissive for microbial attachment.

Curiously, of all samples, tumour was almost completely uninfluenced by location. Rather than from the outside, it is possible that intratumoural conditions, such as oxygen availability, density, necrosis and other factors [67,68] independent of the external conditions are more likely to be the influencing ones. This is, however, a matter for further research.

The major strongpoint of this study is the analysis of the microbiota from four different lung samples covering the major physiologically different environments in NSCLC patient that has not been characterised previously, with the addition of saliva as the sample of oral microbiota. The representation using taxonomical trees also gives better insight into sample's composition, especially important in characterisation of this type of still scarcely defined microbiota. Moreover, this study reports higher numbers of detected OTUs as well as diversity indexes than previously suggested for saliva, non-malignant tissue, tumour and BAL [26,29,30]. The reason could be due to the higher quality of obtained samples (only 6 from 85 samples lower than 1000 reads) and direct sampling of the BAL in the excised lobe. Also, the observed study group and stratification by tumour lobe location were well balanced in various clinical and demographic factors, minimising result's bias.

The major drawback of this study is a low number of subjects, due to strict inclusion criteria for the overall study. The limiting factor was also the decision to obtain BAL directly from the excised lobe to improve the precision in characterisation, but disabling the possibility of sampling the complementary non-tumour lobe or to perform similar in controls due to difference in technique.

5 Conclusion

To our knowledge, this is the first trial that studied oral and lung microbiota from both BAL and three different tissue samples in 18 NSCLC patients. We confirmed that oral and lung microbiota are significantly different both in diversity and in taxonomy. However, we showed that BAL indeed represents a unique microbiota compared to three different tissue microbiota

(from non-malignant, peritumoural and tumour tissue). We found that location of the tumour in upper or lower lobes influenced both oral, BAL and extratumoural microbiota with detection of *Firmicutes* as dominating phylum, but surprisingly, not the one of the tumour. Moreover, few differences were found between tissues, suggesting that these are not conditioned by lobe location and turning attention to other factors for future consideration, such as tumour type, aggressiveness or metastatic changes. Finally, the most intensive changes in microbiota relative to location were seen in peritumoural tissue, possibly reflecting changes in tumoural ECM. Our findings are the first to give essential characteristics and differences within lung microbiota of NSCLC and their susceptibility to tumour lobe location, proposing several possible implications of microbiota in pathology of lung cancer and suggesting potential research directions for better understanding of the lung microbiota-cancer interaction.

6 Declarations

6.1 Ethics approval and consent to participate

The study (and its amendment in June 2018) was approved by the Committee for the Protection of Persons (CPP) Sud-Est VI, Clermont-Ferrand, France, and The French National Agency for Medicines and Health Products Safety (ANSM) (study ref. 2016-A01640-51). The study is registered with the Clinical Trials under ID: NCT03068663. Written informed consent was obtained from all patients before enrolment in the study.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and material

According to the practice of the sponsoring institution (Centre Jean Perrin), all samples will be preserved for 15 years. The datasets generated and/or analysed during the current study are confidential but are available from the corresponding author on reasonable request and after the data sharing process is brought into compliance with GDPR (Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016).

6.3.1 Data sharing

This statement is intended to clarify Centre Jean Perrin's position on data sharing with regards to applicable regulatory frameworks.

6.3.1.1 European regulatory framework

De-identified study data are considered as personal data within the meaning of Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016, known as « GDPR ».

As a result, their transfer places further processors in the scope of GDPR (Art. 2 and 3) and is notably subject to

- precise information of study subjects (Art. 14),
- prior identification of further processors and signature of a transfer agreement (Chapter V),
- data protection impact assessment (Art. 35).

6.3.1.2 French regulatory framework

In addition to GDPR, the transfer of this data by Centre Jean Perrin is a processing in itself that is subject to Chapter IX and XII of the updated Law n°78-17 of 6 January 1978. Within the framework of this law, the data processing is permitted by Centre Jean Perrin's conformity to the MR-001 Code of conduct (Délibération n° 2018-153 du 3 mai 2018 portant homologation d'une méthodologie de référence relative aux traitements de données à caractère personnel mis en œuvre dans le cadre des recherches dans le domaine de la santé avec recueil du consentement de la personne concernée).

In its last update from May 2018, MR-001 was updated to allow transfer to reviewers within certain conditions (Article 2.4).

These conditions can be summarised as follow:

The access to study data by an **independent mandated reviewer** can only be granted through an interface chosen by the data processor (i.e. Centre Jean Perrin, in this context) for the sole purpose of re-analysis or for any other purpose supported by a strong rationale and a project synopsis describing why access to the this study's data (study acronym: MICA) is necessary.

The data processor shall ensure:

- That the chosen sharing platform cannot allow extraction of the data;
- To grant specific personal and differentiated authorizations to access the data;
- That the users be reliably identified
- That state of the art cryptography and security measures are used;
- That an audit trail of accesses is used;

- When data is transferred outside of the EU, that applicable transfer contracts are established;
- That study subjects are informed on the data recipients;
- That the data is used for the sole purpose of reproducing published results;
- That the data is cleaned from any directly identifying data and that the principle of data minimization is applied (i.e. limiting the transfer to data used in the publication).

6.3.1.3 Study Data sharing

As a result, for this trial (acronym: MICA), Centre Jean Perrin can share individual participant data that underlie the results reported in the article under the following conditions:

- Limitation to the data processing to the purpose of independent review of the published results;
- Prior minimization of the data (i.e. restriction to data strictly adequate, relevant and limited to the purpose of the independent review of results);
- Prior identification of the reviewer and authorization from Centre Jean Perrin for a personal access;
- Following the signature of a transfer agreement with Centre Jean Perrin, in conformity with the European Commission's Standard Contractual Clauses;

Access to study data will be assessed, upon written detailed request sent to the project manager Emilie THIVAT: emilie.thivat@clermont.unicancer.fr, from 6 months until 5 years following article's publication.

6.4 Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6.5 Funding

This work was financially supported by the Auvergne Region (France), European Regional Development Fund (FEDER) and company Greentech (Saint-Beauzire, France). The study sponsor was Jean Perrin Centre, Clermont-Ferrand, France.

6.6 Authors' contributions

ABD, EF, JYB, MF and MPV were responsible for the study design. ABD, ED, EF, MF and RB planned the experiments. RB, ED and MF performed the experiments. MF and ABD aided

in interpreting the results. RB analysed the results with the statistical approach devised from IM. RB designed the figures and wrote the manuscript. All authors gave their critical feedback for the final manuscript version.

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8 Additional files

Additional file 1.pdf – contains:

- Figure 1 Most abundant genera in four lung and salivary microbiota relative to tumour lobe location.
- chapter “Additional explanation of differences between lung samples (Main manuscript: Fig. 6)” that adds details on significant differences found in Figure 6

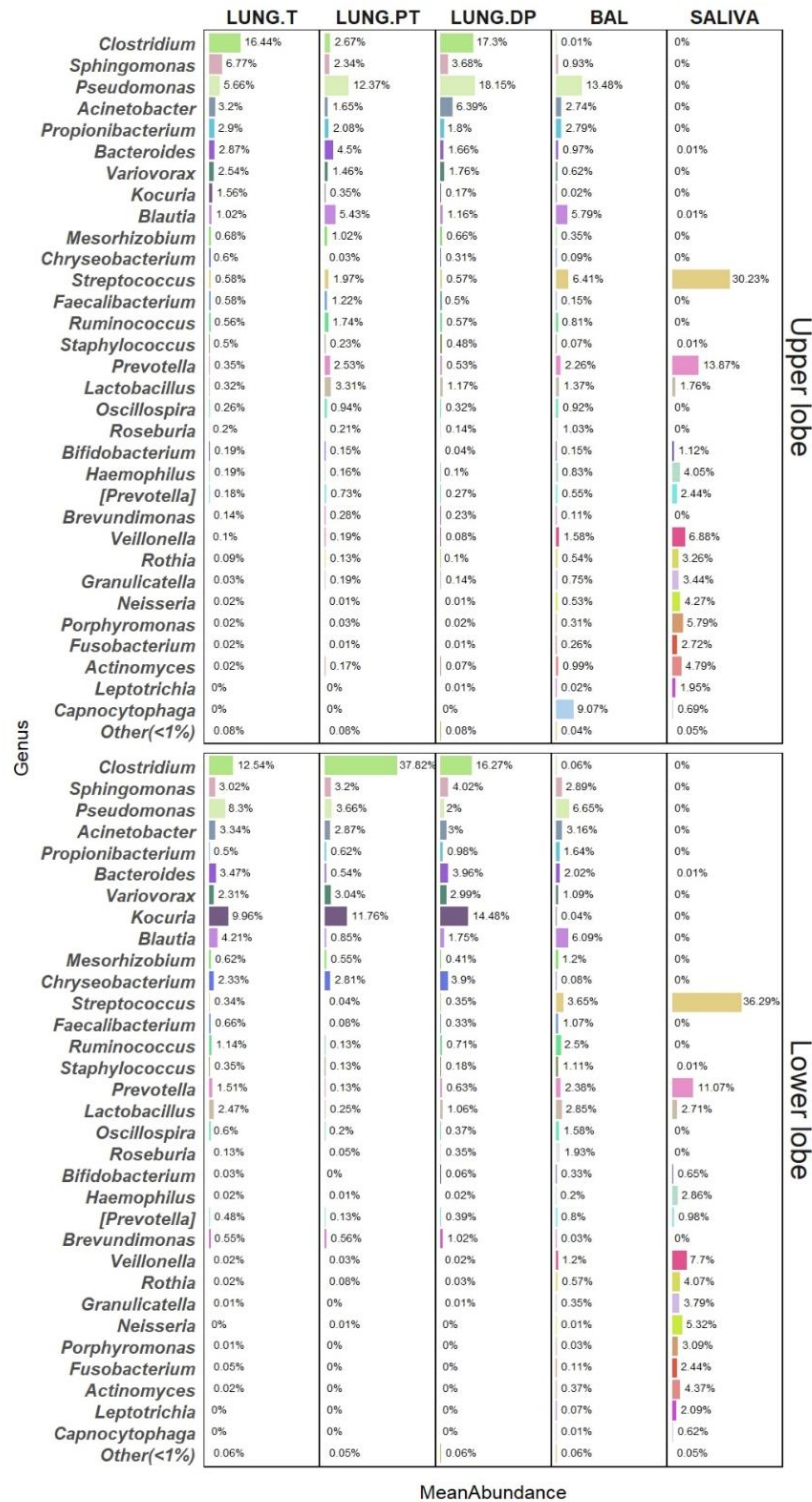


Figure 1 Most abundant genera in the four lung and salivary microbiota relative to tumour lobe location. BAL - bronchoalveolar lavage fluid, LUNG.DP - non-malignant distal piece, LUNG.PT - peritumoural tissue, LUNG.T - tumour.

Additional explanation of differences between lung samples (Main manuscript: Fig. 6)

Except mentioned characteristics, non-malignant tissue and BAL shared similar differences between locations, unlike other two tissues. Peritumoural tissue and BAL were more different in lower (LL) than in upper lobes (UL), seen by significantly increased abundance of members of class *Bacteroidia*, *Coriobacteriia*, overall phylum Firmicutes and orders *Pasteurellales*, *Bifidobacteriales* in LL BAL than in LL peritumoural tissue, not seen in UL. On the other hand, tumour and BAL were more different in UL and represented the richest comparison tree in tissue vs. BAL category (Main manuscript: Fig. 6). Classes *Alphaproteobacteria*, *Cytophagia*, [*Saprospirae*] and orders *Myxococcales*, *Bdelovibrionales* and *Turcibacterales* were significantly more abundant in UL tumour than in BAL, and no difference was seen in LL. On the contrary, phylum *Fusobacteria* and class *Gammaproteobacteria* were both more abundant in UL BAL than in tumour (no diff. in LL).

Additionally, genera *Acinetobacter* and *Dietzia* were more abundant in non-malignant tissue than in peritumoural tissue, while no differences were detected between non-malignant tissue and tumour in UL. Conversely, several taxa were different between UL peritumoural tissue and tumour, including family *Lachnospiraceae* and genera *Actinomyces*, *Dialister* and *Prevotella* more abundant and *Sediminibacterium*, *Phenylobacterium* and family *Myxococcales* less abundant in UL peritumoural tissue. In LL, the differences were very few. Genus *Bifidobacterium* was significantly less abundant in LL peritumoural tissue in the three tissues and family *Microbacteriaceae* in tumour. In addition, class *Erysipelotrichi* was significantly more abundant in LL tumour than in LL peritumoural tissue.

Article 2: Lung microbiota and metastatic lymph nodes

Research Article

Inverse abundance pattern of tumour and extratumoural lung microbiota between non-small cell lung cancer patients with or without metastatic lymph nodes: a cross-sectional clinical study

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Presence of metastatic lymph nodes (LN) is one of the most important negative prognostic markers in NSCLC, characterised by significantly shorter overall survival and increased recidivism. We investigated if this important feature for patient's outcome is significantly associated to local lung microbiota composition, based on four different samples (BAL, non-malignant, peritumoural and tumour tissue). Further, we examined if these two endpoints (presence or absence of the lymph node metastasis) influence local immunity by investigating the composition of the tumour immune infiltrate and Th cell phenotypes in BAL. Finally, we investigated if this immune response is associated to different lung microbiota.

We show that in the presence of metastatic lymph nodes all lung microbiota samples have increased abundance of the phylum *Firmicutes*, while diversity metrics show lower values for the two extratumoural tissue microbiota. We identify 31 genera with inverse patterns in the abundance between tumour and extratumoural tissues, and between patients with and without metastatic LN. Moreover, we show that this inversion is closely related to bacterial respiratory profile. More precisely, identified anaerobic genera are significantly more abundant within the tumours with metastatic lymph nodes, while aerobic bacteria are more abundant in the extratumoural tissues. However, exactly inverse is observed for these same genera in patients without metastatic LN. Therefore, we propose that bacteria “migrate” between the tissues towards the more favourable growth conditions, since tumour environment drastically changes with its progression (increased hypoxia, necrosis, lactate production, lower pH). Moreover, we identify several potential biomarkers in each tissue that might facilitate or improve detection of the LN metastases without imposing LN biopsy. Finally, we show that BAL microbiota does not change significantly between these endpoints, but that it is the only one with significant association with either protumoural or antitumoural immune markers in BAL and in the tumour.

Research Article

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Abstract

Background. Lung cancer is the leading cause of death by cancer worldwide. In non-small cell lung cancer (NSCLC) as the most common type, metastatic changes on lymph nodes (LN) are associated with shorter survival and worse prognosis. Unlike in other cancers, interaction of lung microbiota with various origin and lung cancer is not well documented. To investigate the relation between lung microbiota, local immune response and metastatic LN, we conducted an observational cross-sectional study in 17 NSCLC patients. Lung microbiota was analysed in bronchoalveolar lavage fluid (BAL) (direct washing of the excised lobe) and three tissues: non-malignant, peritumoural and tumour tissue. Accompanying immune status was assessed as tumour infiltrating lymphocytes and T helper and neutrophil profiles in BAL.

Results. We found that phylum *Firmicutes* replaced *Proteobacteria* as dominating in tumour and peritumoural tissue in the presence of metastatic LN. Next, anaerobic genera were significantly more abundant in tumours with metastatic LN, while aerobic genera were significantly more abundant in extratumoural tissues. However, the inverse was noted in patients without metastatic LN. 8 genera from three tissues were identified as promising prognostic biomarkers of the presence of the metastatic LN (combined AUC of 86.1%, 90.6% and 92.9% per tissue, respectively). From the four analysed lung communities, only BAL microbiota had high association with local immune response (both intratumoural and in the BAL). Moreover, genera positively associated with either anti-tumour or pro-tumour markers shared the same taxonomic lineage as identified anaerobic (mostly order *Clostridiales*) and aerobic tissue genera (mostly class *Alphaproteobacteria*), respectively.

Conclusions. We evidenced a close connection between metastatic status of LN and tissue lung microbiota (extratumoural and tumoural) in NSCLC patients, but also the one between BAL microbiota and local profile of immune cells. Our findings encourage further investigation of the interaction between lung microbiota from multiple sites and tumour immunity and environment. Also, the potential clinical application in detection of the metastatic LN through bacterial pattern in NSCLC patients will require confirmation on larger patient population.

Trial registration. ClinicalTrials.gov ID: NCT03068663. Registered February 27, 2017. <https://clinicaltrials.gov/ct2/show/NCT03068663>

Key words:

Lung microbiota, lung cancer, non-small cell lung cancer, metastatic lymph nodes, bronchoalveolar lavage, tumour, lung tissue, non-malignant tissue, peritumoural tissue

1 Background

Lung cancer is a leading cause of death by cancer worldwide, the 1st most frequent cancer in men and the 3rd most frequent in women [1]. It is markedly more frequently diagnosed in older populations (over 65 years), with non-small cell lung cancer (NSCLC) counting for 85-90% of all lung cancer cases [2].

The genetic aetiology of NSCLC is already extensively described [3], but the link between lung cancer and lung microbiota remains scarcely explored [4], despite the growing interest for cancer-microbiota interaction and the emergence of the gut-lung axis theory [5–7] in the last decade. The reason partially lies in the long-lasting perception of NSCLC principally, but not exclusively, as a direct consequence of gene alteration due to various external or internal factors [2]. Recently, however, a clear connection was established between chronic inflammation and lung cancer development in the studies of chronic obstructive pulmonary disease (COPD) [8,9]. Moreover, as well as the role of microbiota in these pathologies [10–14], the role of lung microbiota in lung cancer was suggested as one of the important questions to consider [15]. Several animal models have recently proved that the presence, absence or dysbiosis (e.g. due to antibiotics) of lung microbiota could influence both lung tumour growth [16,17] and immune response in the tumour bed [18]. Moreover, a connection was found between enrichment of the lower airways by oral or supraglottic taxa and increased inflammation and Th17 recruitment in lung cancer patients that could stimulate pathways involved in tumour progression [19–21].

The studies involving human subjects remain, however, a few numbered, with the invasiveness of the sampling methods as the major inconvenience for lung microbiota research. So far, most of the studies on lung microbiota were done on asthmatic or COPD patients by analysis of sputum or bronchoalveolar lavage fluid (BAL) obtained by bronchoscopy [5,10,14,22], often used in a routine patient follow-up. Even though these techniques are less invasive than tissue sampling and therefore more readily performed, they have a higher risk of contamination of already low biomass samples by upper airways microbiota [23,24]. Also, BAL is likely to represent lung microbiota found within bronchia that might differ from lung microbiota of the lung tissue. In current studies on lung cancer microbiota [21,25–30], only two included analysis of tissue samples. Consequently, microbiota of the tumour and non-malignant tissue in NSCLC

and its connection to tumour-specific or local immune response have still not been entirely investigated.

In NSCLC treatment, metastatic changes on lymph nodes (LN) are a major prognostic factor [31,32], and are defined by TNM classification (i.e. tumour staging) [33]. The presence of metastatic changes on LN has been related to a significant decrease in overall survival and response to therapy [33], and is therefore directly implicated in cancer progression and outcome. However, characterisation of the lung microbiota in the light of metastatic LN changes has still not been addressed. This knowledge could yield a better understanding of the tumour microbiological environment and potential involvement in metastatic behaviour of the tumour.

To broaden insight into both of these matters, we conducted a cross-sectional pilot study in 17 NSCLC patients with primary non-metastatic lung tumours eligible for surgery as a part of our project [34]. As the first objective, this study determined whether the difference in clinical tumour stage based on metastatic changes in LN was accompanied by an underlying difference in the lung microbiota of various origins (BAL, tumour, peritumoural tissue, non-malignant “healthy” tissue). The second objective of the study was to investigate the connection between these microbiota and the local immune response (tumour lymphocytic infiltration, immune cell phenotypes in BAL) in these patients.

2 Results

2.1 Study population

Eighteen patients were prospectively included in the study. Ultimately, lung samples were obtained from seventeen patients, which were kept for further analysis. Following the post-resection TNM classification by a pathologist (Table 1), the patients were divided into two groups for the purpose of this study: Stage I,II patients without metastatic LN, and Stage III patients with metastatic LN. The groups’ characteristics are presented in Table 2. Body mass index (BMI) was the only characteristic that differed significantly between the two groups and corresponded to reported weight loss from Stage III patients. The Chi-square test showed that both groups were balanced in terms of tumour location, smoking status, tumour type and sex (data not shown).

Table 1 Post-surgical TNM classification

pTx	pNx	Tumour type	Tumour size (cm)	Stage group
T1	N0	carcinoid	3.0	I,II
T1	N0	ADK	2.5	I,II
T1	N0	ADK	0.6	I,II
T1	N0	ADK	1.7	I,II
T2	N0	ADK	4.5	I,II
T2	N0	ADK	3.8	I,II
T2	N0	ADK	3.0	I,II
T2	N0	SCC	4.0	I,II
T3	N0	carcinoid	5.0	I,II
T2	N2	ADK	3.0	III
T2	N2	ADK	3.0	III
T2	N2	ADK	2.4	III
T3	N1	ADK	2.0	III
T4	N1	ADK	2.3	III
T4	N1	SCC	8.0	III
T4	N1	SCC	9.5	III
T4	N2	SCC	7.0	III

ADK – adenocarcinoma; SCC – squamous cell carcinoma

Table 2 Characteristics of patients included in the study

Tumour stage:	Stage I, II	Stage III	<i>p</i>
Total no. of patients	9	8	
Stage I/II/III	7/2/0	0/0/8	
ADK/SCC/carcinoid	6/1/2	5/3/0	
Male/female	6/3	6/2	
Smoker/ex-smoker/never-smoker	2/7/1	0/7/1	
Pack-year (smokers and ex-smokers)	32 ± 23	31 ± 20	0.809
Age (years)	66 ± 10	71 ± 4	0.358
BMI	26.4 ± 3.6	23.6 ± 1.8	0.038
FEV ₁ (% of expected value)	96 ± 8	100 ± 15	0.606

2.2 Lung microbiota and tumour stages

2.2.1 Diversity differed relative to Stage group, with domination of phylum Firmicutes in tumour (LUNG.T) and peritumoural tissue (LUNG.PT) of Stage III patients

Four lung samples characterised throughout the study were: BAL, non-malignant Distal tissue Piece (LUNG.DP), Peritumoural Tissue (LUNG.PT) and Tumour (LUNG.T).

Tumour and non-malignant tissue had significantly different beta diversity between Stage groups (Fig. 1a, 1b) based on weighted (wUF) and unweighted UniFrac (uwUF) distance, respectively. Even though initially no differences were observed between tissue samples (data not shown), tumour and peritumoural tissue (two closest tissues by sampling location) were significantly different in Stage I, II (uwUF, Fig. 1c), while tumour and non-malignant tissue (two most distant tissues by sampling location) were significantly different in Stage III (uwUF, Fig. 1d).

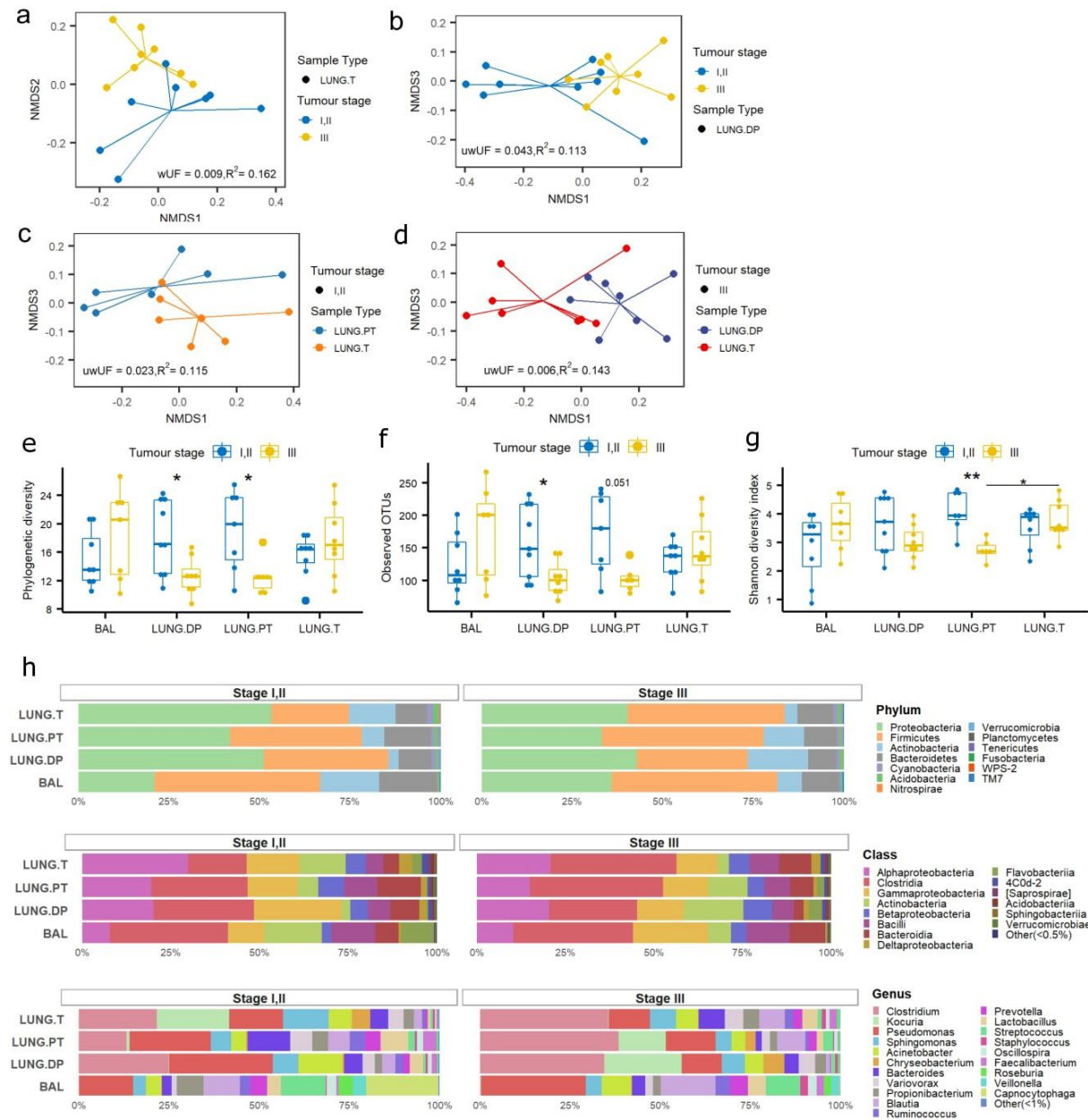


Figure 1 Characterisation of the microbiota in lung samples. Beta diversity (presented by NMDS) in (a) tumour (LUNG.T) between Stage I,II (N = 8) and Stage III (N = 8) based on wUF, (b) non-malignant tissue (LUNG.DP) between Stage I,II (N = 9) and Stage III (N = 8) based on uwUF, (c) Stage I,II between tumour (LUNG.T) (N = 8) and peritumoural tissue (LUNG.PT) (N = 7) based on uwUF, (d) Stage III between LUNG.T (N = 8) and LUNG.DP (N = 8) based on uwUF. Alpha diversity was analysed between Stage I,II and Stage III for each sample type: (e) phylogenetic diversity, (f) observed OTUs, (g) Shannon diversity index. Alpha diversity was also analysed between samples within each Stage group, and the only significant result was in Shannon index for Stage III between LUNG.T and LUNG.PT (g). (h) Relative abundance relative to the sample type and Stage group in the phylum, class and genus level. (e-h) Sample numbers were in Stage I,II: LUNG.T (N = 8), LUNG.PT (N = 7), LUNG.DP (N = 9), BAL (N = 8), and in Stage III: LUNG.T (N = 8), LUNG.PT (N = 7), LUNG.DP (N = 8), BAL (N = 7). Difference in beta diversity was tested by adonis function (vegan) based on 999 permutations. Difference in alpha diversity between Stage groups was tested by unpaired Wilcoxon test and between sample types within group by Kruskal-Wallis with BH correction for multiple comparison.

BAL – bronchoalveolar lavage fluid; LUNG.DP – non-malignant distal piece, LUNG.PT – peritumoural tissue; LUNG.T – tumour. *: $p < 0.05$, **: $p < 0.01$

Inversely, BAL was initially different from each tissue sample (all differences at least $p < 0.05$ based on both wUF and uwUF) (data not shown). However, in within-group analysis there was no significant difference between BAL and peritumoural tissue for both groups in wUF (Stage I,II: $p = 0.069$, $R^2 = 0.125$; Stage III: $p = 0.174$, $R^2 = 0.117$) and only in Stage III for uwUF (Stage I,II: $p = 0.027$, $R^2 = 0.117$; Stage III: $p = 0.082$, $R^2 = 0.135$). Interestingly, in Stage III increased similarity was observed between BAL and tumour (uwUF $p = 0.061$, $R^2 = 0.126$; Stage I,II: uwUF $p = 0.001$, $R^2 = 0.199$).

Several alpha diversity metrics (Fig. 1e-g) were significantly lower in Stage III patients both in non-malignant and peritumoural tissue. Inversely, in Stage III, both in BAL and tumour, Shannon diversity showed tendency for higher values (Fig. 1g). In addition, in Stage III, tumour had significantly higher Shannon diversity than peritumoural tissue (Fig. 1g).

Fig. 1h represents the relative abundance of microbiota in phylum, class and genus level, respective to the sample type and tumour stage group. In Stage I,II, the lung tissue samples were dominated by two phyla: *Proteobacteria* (~50%, especially classes *Alphaproteobacteria* and *Gammaproteobacteria*) and *Firmicutes* (~30%, class *Clostridia*). Phyla *Actinobacteria* (class *Actinobacteria*) and *Bacteroidetes* (class *Bacteroidia*) followed. In stage III, however, tumour and peritumoural tissue were dominated by phylum *Firmicutes* (~44%) and *Proteobacteria* was the 2nd most abundant (~37%). In non-malignant tissue, there was no difference in dominating phyla between stages. However, in Stage III abundance of *Proteobacteria* was lower in favour of *Actinobacteria* (~17%).

At the genus level (Fig. 1h), tissue samples shared dominating genera. *Clostridium* (*Firmicutes*), *Pseudomonas* (*Gammaproteobacteria*), *Kocuria* (*Actinobacteria*), *Bacteroides* (*Bacteroidetes*) *Sphingomonas* (*Alphaproteobacteria*), *Acinetobacter* (*Gammaproteobacteria*) and *Blautia* (*Firmicutes*) were among the more abundant genera in both groups. Interestingly, the *Clostridium* genus, which in Stage I,II was balanced in abundance with either *Pseudomonas* or *Kocuria* depending on the tissue, was ~2-fold more abundant in Stage III and representing the most abundant genus (~30%).

In Stage I,II (Fig. 1h), BAL was dominated by *Firmicutes* (~45%, class *Clostridia*), followed by almost equal abundances of *Proteobacteria* (~20%, class *Gammaproteobacteria*), *Actinobacteria* (~16%, class *Actinobacteria*) and *Bacteroidetes* (~16%, class *Flavobacteriia*).

In Stage III, however, *Proteobacteria* (~36%, classes *Alphaproteobacteria* and *Gammaproteobacteria*) was more abundant than *Bacteroidetes* and *Actinobacteria*. Even though in Stage I,II *Capsocytophaga* (~9%) was the most abundant genus, followed by *Pseudomonas*, *Streptococcus* and *Blautia* (~6% each), Stage III was dominated by *Pseudomonas* (~15%), *Blautia* (~8%) and *Streptococcus* (~6%). Interestingly, *Clostridium*, found as most abundant in lung tissues, was not in the first 50 most abundant genera in BAL in either of the groups.

2.2.2 Anaerobic and aerobic bacteria showed inverse abundance profiles between tumour and extratumoural tissue

Figure 2 shows only taxa on the levels of class and genus whose abundance was significantly different between two Stage groups. Difference in abundance is presented as log₂-fold change. Within each bar there is a significance level and beside the bar is a number representing the rank of importance of the difference as reported by random forest algorithm. BAL samples were non-significantly different on selected taxonomic ranks, so they were excluded from graphics.

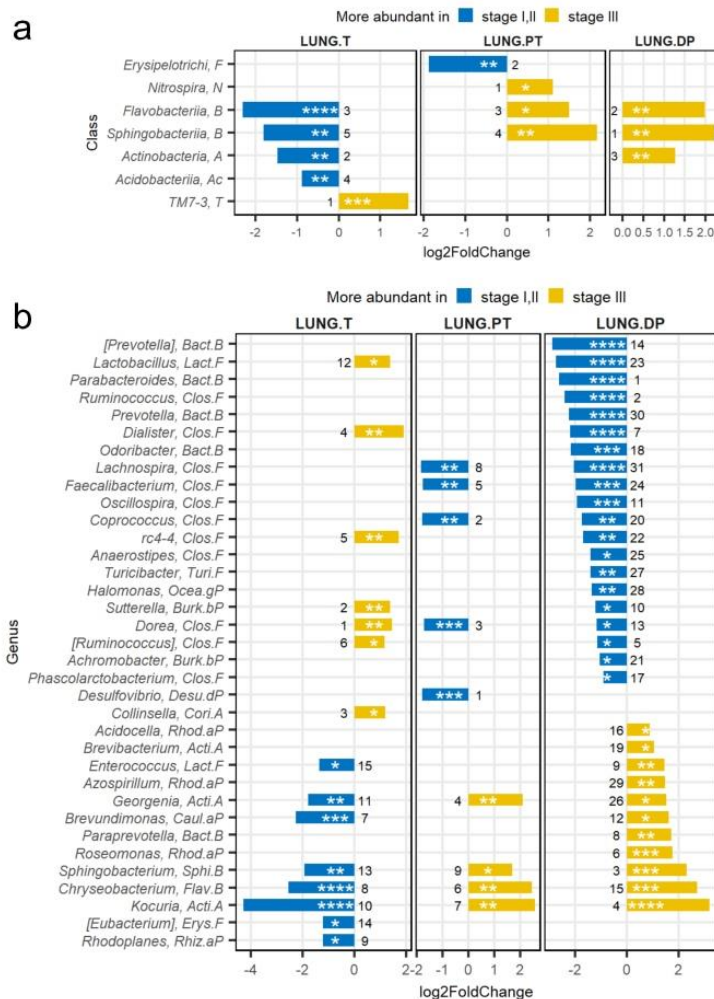


Figure 2 Differential abundance of lung tissue microbiota between Stage groups. (a) Differential abundance on the class level. (b) Differential abundance on the genus level.

Asterisk within the bars represents the adjusted significance (BH) of differential abundance tested by Wald's test of significance of coefficients in a negative binomial linearized model (microbiomeSeq). Sample numbers were in Stage I,II: LUNG.T (N = 8), LUNG.PT (N = 7), LUNG.DP (N = 9), and in Stage III: LUNG.T (N = 8), LUNG.PT (N = 7), LUNG.DP (N = 8). The numbers beside the bars are ranks of importance of the detected difference, as detected by random forest classifier. Following full taxa names are corresponding order (4 letters) and phylum (1 capital letter). Orders: *Acti* – *Actinomycetales*, *Bact* – *Bacteroidales*, *Burk* – *Burkholderiales*, *Caul* – *Caulobacteriales*, *Clos* – *Clostridiales*, *Cori* – *Coriobacteriales*, *Desu* –

Desulfovibrionales, *Erys – Erysipelotrichales*, *Flav – Flavobacteriales*, *Lact – Lactobacillales*, *Ocea – Oceanospirillales*, *Rhiz – Rhizobiales*, *Rhod – Rhodospirillales*, *Sphi – Sphingobacteriales*, *Turi – Turicibacterales*. Phyla: *A – Actinobacteria*, *B – Bacteroidetes*, *F – Firmicutes*, *P – Proteobacteria* (classes *aP – Alphaproteobacteria*, *bP – Betaproteobacteria*, *gP – Gammaproteobacteria*, *dP – Deltaproteobacteria*). [*Prevotella*] is from the family *Paraprevotellaceae*, and *Prevotella* from *Prevotellaceae*. [*Ruminococcus*] is from the family *Lachnospiraceae*, and *Ruminococcus* from *Ruminococcaceae*. BAL – bronchoalveolar lavage fluid; LUNG.DP – non-malignant distal piece, LUNG.PT – peritumoural tissue; LUNG.T – tumour. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

The eye-catching feature was that the log₂ profiles showed strong similarities for peritumoural and non-malignant tissue, and were inverse to tumour for each shared taxa (Fig. 2a, 2b). This was observed both on class and genus level. Classes *Sphingobacteriia*, *Flavobacteriia* and *Actinobacteria* were significantly more abundant in non-malignant and/or peritumoural tissue of Stage III patients, but less abundant in Stage III tumour (Fig. 2a). Tumour had two unique differences: classes TM7-3 and *Acidobacteria*. TM7-3 ranked as the most important difference (random forest), more abundant in Stage III (0% vs 0.13%), while *Acidobacteria* was more abundant in Stage I,II. Likewise, two unique differences could be found in peritumoural tissue: *Nitrospira* (1st ranked by random forest) more abundant in Stage III, and *Erysipelotrichi* (2nd ranked) more abundant in Stage I,II.

The total of 31 genera were found significantly different between stages (Fig. 2b), with the highest number in non-malignant tissue. Markedly, an exclusive polarity was seen between the “lower” and “upper” part of the figure when observing higher taxonomic levels of listed genera (Fig. 2b – abbreviations next to the genus name). The “upper” part included listed genera from [*Prevotella*] to *Collinsella*), which were more abundant in Stage III tumour and, inversely, in peritumoural/non-malignant tissue of Stage I,II patients. They belonged to orders *Clostridiales* and *Turicibacterales* (phylum *Firmicutes*), *Bacteroidales* (*Bacteroidetes*), *Coriobacteriales* (*Actinobacteria*), *Burkholderiales* (*Betaproteobacteria*), *Oceanospiralles* (*Gammaproteobacteria*) and *Desulfovibrionales* (*Deltaproteobacteria*). Conversely, the “lower” part of the figure included genera from orders *Actinomycetales* (*Actinobacteria*), *Rhodospirillales*, *Rhizobiales* and *Caulobacterales* (*Alphaproteobacteria*), *Sphingobacteriales* and *Flavobacteriales* (*Bacteroidetes*) and *Erysipelotrichales* (*Firmicutes*).

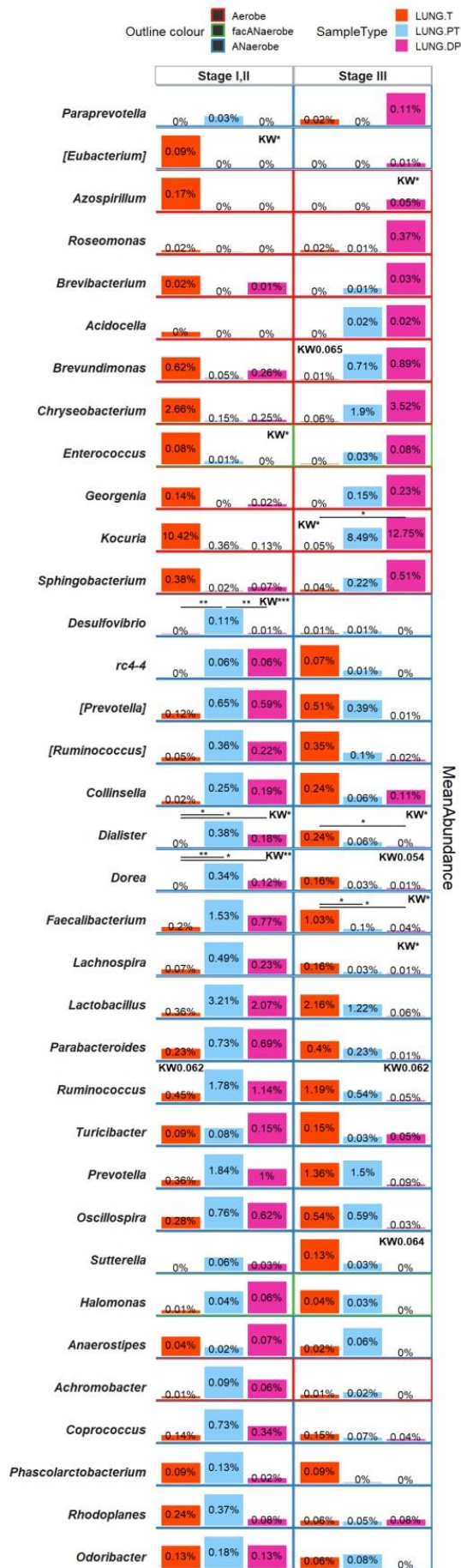


Figure 3 Relative abundance of genera and their respiratory profiles in lung tissues relative to Stage group. Genera from Fig. 2b were analysed for difference in relative abundance between tissue samples within each Stage group. The test used was Kruskal-Wallis with BH correction for multiple comparison. Outline of individual facets represents respiratory profile of given bacteria. facANaerobe – facultative anaerobe. [Prevotella] is from the family *Paraprevotellaceae*, and *Prevotella* from *Prevotellaceae*. [Ruminococcus] is from the family *Lachnospiraceae*, and *Ruminococcus* from *Ruminococcaceae*. BAL – bronchoalveolar lavage fluid; LUNG.DP – non-malignant distal piece, LUNG.PT – peritumoural tissue; LUNG.T – tumour. *: p < 0.05, **: p < 0.01, ***: p < 0.001

We could see that not all of the classes or phyla detected in Fig. 2a were found in genera from Fig. 2b. This was because certain classes, such as *TM7-3*, do not have classified genera. However, on the family level, *F16* from the phylum *TM7* was also 1st ranked as the most important difference in tumour (data not shown).

For 31 significantly different genera between stages (Fig. 2b), we looked more closely at relative abundance for each of these in the three tissues and their biological properties (respiratory type, motility) (Fig. 3). Despite not being significantly different between stages for each tissue (Fig. 2b), the majority of genera followed the inversion principle (if more abundant in tumour, less abundant in peritumoural/non-malignant tissue, and *vice versa*) (Fig. 2b). Depending of the sense of inversion, we deciphered two main groups with mutually inverse abundance change between Stage groups (Fig. 3). The first group contained genera that

were more abundant in tumour in Stage I,II (low in peritumoural/non-malignant tissue), but in Stage III were, inversely, more abundant in peritumoural/non-malignant tissue (low in tumour). This involved mostly genera in the first part of the Figure 3 (from [*Eubacterium*] to *Sphingobacterium*), and included *Chryseobacterium* and *Kocuria* with higher part in overall abundance (Fig. 3). Moreover, these bacteria had in majority aerobic or facultative anaerobic respiration (red and green outline, Fig. 3). The second group involved genera that in Stage I, II were more abundant in peritumoural/non-malignant tissue (low in tumour), but in Stage III were more abundant in tumour. This included mostly genera in the middle of Figure 3 (from *Desulfovibrio* to *Halomonas*) with higher abundant *Lactobacillus*, *Faecalibacterium*, *Ruminococcus* and *Prevotella* (Fig. 3). Considering the respiratory metabolism, all bacteria in this group were obligate anaerobes. Interestingly, in both groups, certain genera in peritumoural tissue had the same abundance or with very little change in both Stage groups (e.g. *Prevotella* or *Azospirillum*). Also, certain genera did not entirely follow the principle of inverse change in abundance (Fig. 3, from *Anaerostipes* until *Odoribacter*). Nevertheless, the pattern and respiratory profile of these genera were more similar to the second than to the first group (i.e. anaerobic). Finally, we also looked at active motility organs in these bacteria, but we found that only 25% possessed flagella with no difference depending on the group.

2.2.3 Combination of genera as potential biomarkers of metastatic lymph nodes

We used receiver operating characteristic (ROC) analysis to assess genera from Figure 3 as potential biomarkers in determination of metastatic LN (Fig. 4). In each tissue, several genera already individually showed high percentage of area under the ROC curve (AUC), especially *Dorea* and *Coprococcus* in peritumoural tissue with 90.9% and 89.8%, respectively. However, a combination of several genera in each tissue increased this already high percentage. Genera *Parabacteroides*, *Ruminococcus* (family *Ruminococcaceae*), and *Oscillospira* in non-malignant tissue (LUNG.DP), *Dorea*, *Coprococcus* and *Lachnospira* in peritumoural tissue (LUNG.PT), and *Dialister* and [*Ruminococcus*] (family *Lachnospiraceae*) in tumour (LUNG.T), represented the combinations with the highest predictive power of 86.1%, 92.9% and 90.6% AUC per tissue, respectively.

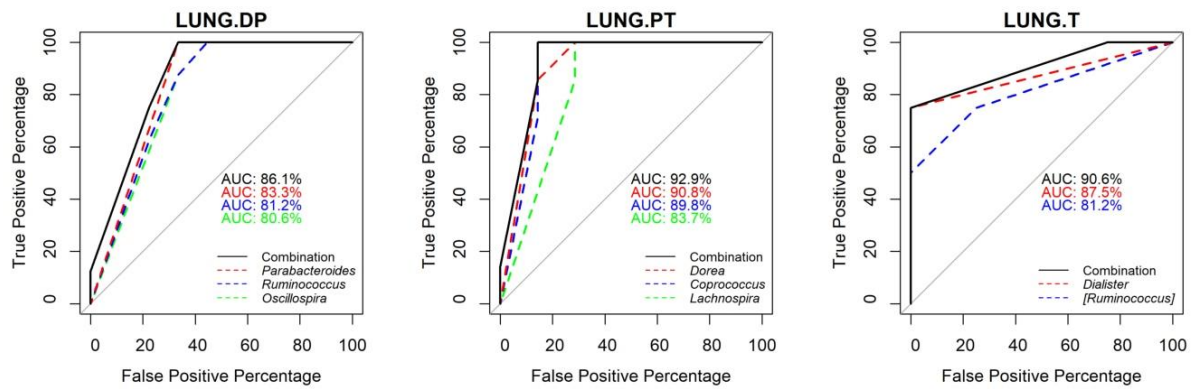


Figure 4 Potential biomarkers for metastatic lymph nodes in three lung tissues. ROC analysis was performed on individual genera detected as significantly different between Stage groups for each tissue in Fig. 2b. Genera with AUC higher than 80% were included for combination analysis. Only combinations with highest AUC percentage were selected and presented in the Figure for each lung tissue along with individual curves. BAL – bronchoalveolar lavage fluid; LUNG.DP – non-malignant distal piece, LUNG.PT – peritumoural tissue; LUNG.T – tumour; AUC – area under the curve

2.3 Tumour-infiltrating lymphocytes and microbiota

2.3.1 Stage groups without difference in tumour-infiltrating lymphocytes (TILs)

Composition of the tumour lymphocytic infiltrate was evaluated by quantity of CD8⁺ (cytotoxic), FOXP3⁺ (regulatory, Treg) and PD-1⁺ T lymphocytes, as well as of CD20⁺ B lymphocytes. In addition, expression of an important immune checkpoint marker, PD-L1, was evaluated both on tumour-infiltrating lymphocytes and tumour cells. No significant difference was seen between tumour stages in any of the TIL subpopulations (Fig. 5a), or in their ratios, CD8⁺/CD20⁺ and CD8⁺/FOXP3⁺ (Additional file 1: Figure 1a). Both groups equally consisted of high- and low-infiltrated tumours. PD-1 and PD-L1 markers were expressed only in a few tumours and did not co-occur in the same tumour nor were they associated with one of the Stage groups (data not shown).

2.3.2 BAL microbiota with the most associations to tumour-infiltrating lymphocytes (TILs)

We next examined the association of microbiota in the tumour or its surroundings with the TILs. Eighteen genera significantly correlated with at least one immune marker (Fig. 5b, Additional file 1: Figure 1b). Correlation profiles were very similar between individual immune markers but not between samples. Surprisingly, not tumour but BAL microbiota showed the highest number of significant correlations with TILs (Fig. 5b). Genera *Dietzia*, *Clostridium*, *Coprococcus*, *Ruminococcus*, *Akkermansia*, *Oscillospira*, *Bacteroides*, *Collinsella*, and *[Prevotella]* had significant positive and *Haemophilus* and *Mesorhizobium* significant negative

correlation with each of TILs' subgroups. On the contrary, correlations in tissue microbiota were mostly negative, and did not follow the trend in BAL. The exception was a significant positive correlation for *Dietzia* seen in non-malignant tissue, similar to BAL. Otherwise, in non-malignant tissue, *Lactobacillus* was significantly negatively correlated, and in tumour significant positive correlation was only with *Sediminibacterium* and *Rhizobium*.

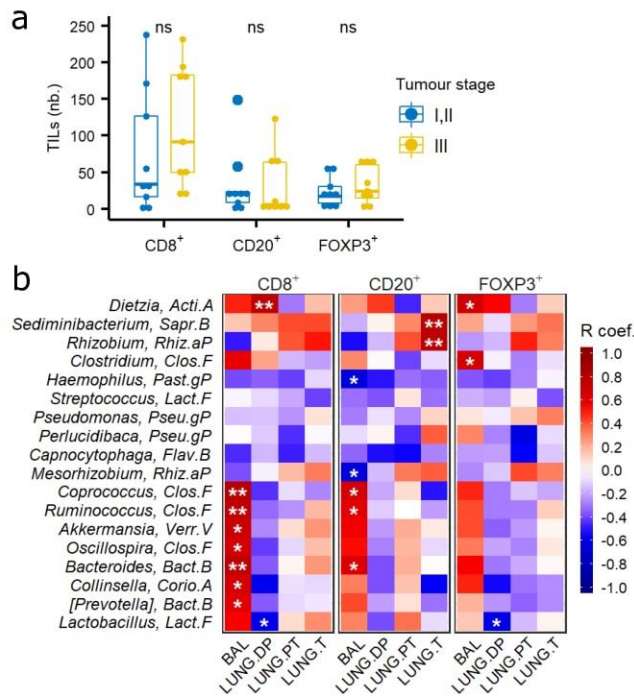


Figure 5 TILs in tumour stages and correlation with lung microbiota. (a)

Difference in no. of tumour-infiltrated lymphocytes (TILs) relative to the tumour stage. (b) Spearman's correlation between microbiota of four lung samples with two TILs ratios and their individual markers. Colour represents correlation coefficient. Shown significance levels are adjusted p-values based on BH correction. Only genera with at least one significant entry are presented. Sample numbers for correlation were: LUNG.T (N = 16), LUNG.PT (N = 14), LUNG.DP (N = 17), BAL (N = 15). TILs – tumour infiltrated lymphocytes. Following full taxa names are corresponding order (4 letters) and phylum (1 capital letter). Orders: *Acti* – Actinomycetales, *Bact* – Bacteroidales, *Clos* – Clostridiales, *Corio* – Coriobacteriales, *Flav* – Flavobacteriales, *Lact* – Lactobacillales, *Past* – Pasteurellales, *Pseu* – Pseudomonadales, *Rhiz* – Rhizobiales, *Sapr* – Saprospirales, *Verr* – Verrucomicrobiales. Phyla: *A* – Actinobacteria, *B* – Bacteroidetes, *F* – Firmicutes, *P* – Proteobacteria (classes *aP* – Alphaproteobacteria, *gP* – Gammaproteobacteria), *V* – Verrucomicrobia. [*Prevotella*] belongs to the family *Paraprevotellaceae*. BAL – bronchoalveolar lavage fluid; LUNG.DP – non-malignant distal piece, LUNG.PT – peritumoural tissue; LUNG.T – tumour. *: $p < 0.05$, **: $p < 0.01$

Interestingly, bacteria that positively correlated with an increased count of TILs' in BAL belonged to phyla *Firmicutes* (order *Clostridiales*), *Actinobacteria* (orders *Coriobacteriales*, *Actinomycetales*) and *Bacteroidetes* (order *Bacteroidales*). Inversely, negatively-correlating genera were mostly from phyla *Proteobacteria* (classes *Alphaproteobacteria* and *Gammaproteobacteria*) and *Bacteroidetes* (orders *Saprospirales* and *Flavobacteriales*).

Furthermore, certain genera that negatively correlated with individual markers showed significant positive correlation with ratios (Additional file 1: Figure 1b). This was, however, not due to true stimulation of the immune response but rather due to less negative correlation with one of the markers, and was not taken as a relevant result.

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2.4 Phenotyping of BAL immune cells and their association to lung microbiota

2.4.1 Th17 higher in BAL of Stage III patients

Next, we looked at difference in percentage of helper T lymphocytes (Th) and polymorphonuclear neutrophil (N) subtypes between Stage groups in samples of BAL. Neutrophil subtypes showed no significant difference between the two Stage groups (Fig. 6a). In CD4⁺ Th, the only difference was observed for Th17 cells (Fig. 6b), found significantly higher in the Stage III group, although overall percentage remained low in both groups. Consequently, both ratios Th1/Th17 and Treg/Th17 were lower in Stage III (Treg/Th17 significantly) (Fig. 6c). Interestingly, the positive correlation between Treg and Th17 was present in the Stage III group, with the similar tendency in Stage I,II group (Fig. 6d).

2.4.2 Pro and anti-tumour markers show medium to strong correlation with BAL microbiota

Similarly, as with the tumour immune infiltrate, we examined the association of immune cells in BAL and microbiota from lung samples. All four samples were analysed, but only BAL showed a clear clustering and significant correlations. Figure 6e therefore shows association between genera in BAL and BAL immune cells with at least one correlation higher than $R = 0.4$.

We could decipher three groups of associations: group 1, from *Oscillospira* to *Ruminococcus*, group 2, from *Haemophilus* to *Atopobium*, and group 3, from *Novosphingobium* until *WAL_1855D* (Fig. 6e). Genera from group 1 positively correlated with Th1, Th2, Th1/Th17, Th1/Treg, Th1/Th2 and inactive circulating and tethering neutrophils (N). These markers are most often considered as a part of anti-tumour response [35]. Inversely, they were negatively correlated with Treg, Th17, activated N and Th17/Treg ratio (significantly for genus *Roseburia* and *Bacillus*), considered as markers of pro-tumour response [35]. Group 3 was the exact opposite of group 1, while group 2 was found in between. Similar to group 1, group 2 had positive associations with Th1-associated markers, but had negative associations with Th2 and circulating N (as in group 3).

Markedly similar to associations observed with TILs, association groups with immune cells in BAL divided genera by their higher taxonomic levels. So, genera in group 1 belonged principally to phyla *Firmicutes* (order *Clostridiales*) and *Bacteroidetes* (*Bacteroidales*), while

in group 3, 12 of 18 genera were from phylum *Proteobacteria*. The additional six genera were from either phylum *Firmicutes* or *Bacteroidetes* (but orders *Saprospirales* and *Flavobacteriales*). Group 2 was again in between, with members from phyla *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Fusobacteria*.

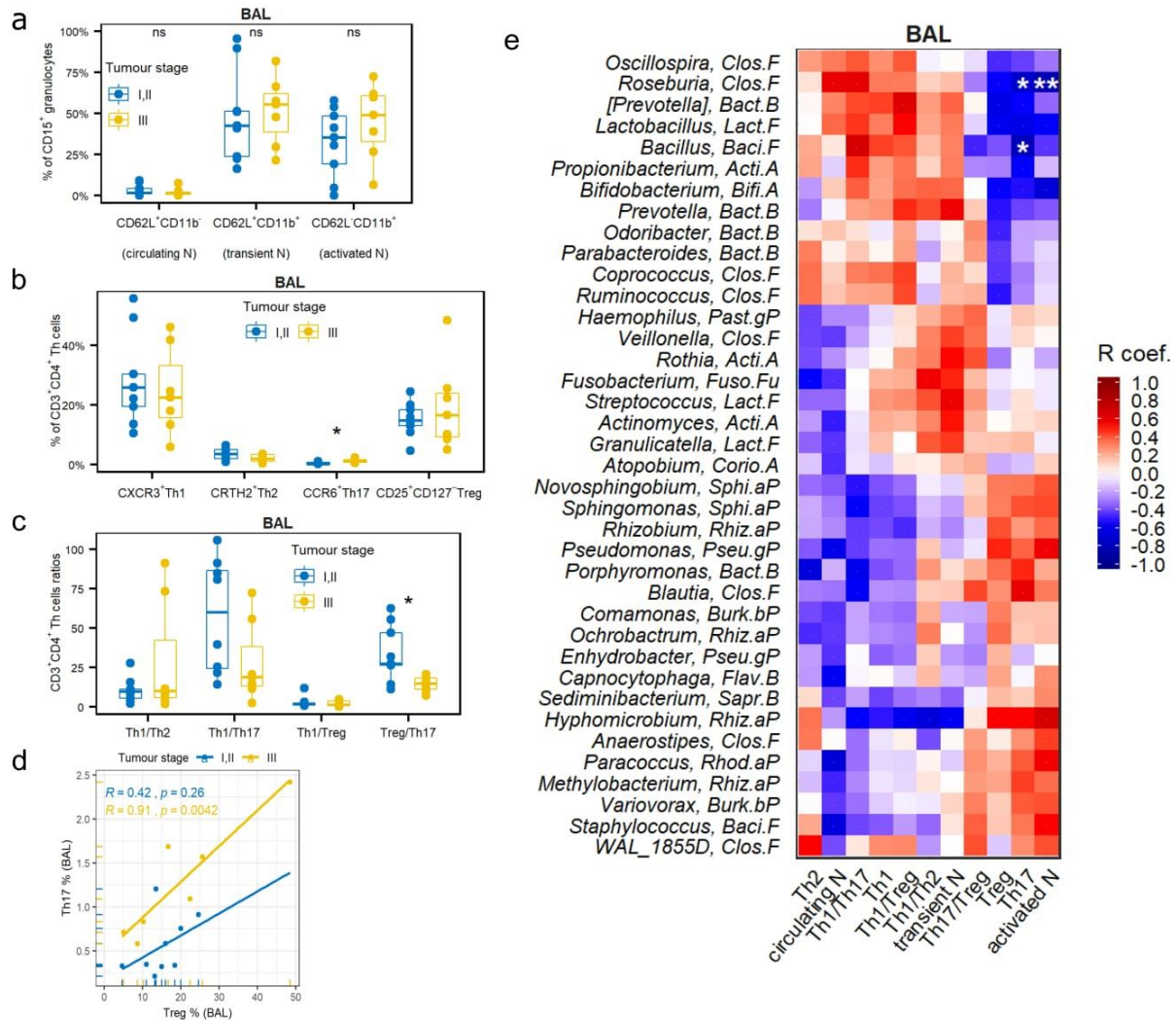


Figure 6 Immune phenotypes in Stage groups and correlation with microbiota in BAL. **a** Comparison of neutrophil subpopulations in BAL between Stage groups. **b** Comparison of CD3⁺CD4⁺ helper T cells in BAL between Stage groups. **c** Comparison of Th ratios between Stage groups in BAL. **d** Spearman's correlation between percentage of Th17 and Treg in BAL relative to Stage group. In subfigures **a**, **b** and **c**, the test used was unpaired Man-Whitney with BH correction for multiple group comparison. **e** Heatmap represents Spearman's correlation between immune profiles in BAL with genus level of the microbiota in BAL. Colour represents correlation coefficient. Shown significance levels are adjusted p-values based on BH correction for multiple comparison. Only genera with $R > 0.4$ (considered as medium strong correlation) are presented. Number of samples: BAL (N = 14). Following full taxa names are corresponding order (4 letters) and phylum (1 capital letter). Orders: *Acti* – Actinomycetales, *Baci* – Bacillales, *Bact* – Bacteroidales, *Bifi* – Bifidobacteriales, *Burk* – Burkholderiales, *Clos* – Clostridiales, *Corio* – Coriobacteriales, *Flav* – Flavobacteriales, *Fuso* – Fusobacteriales, *Lact* – Lactobacillales, *Past* – Pasteurellales, *Pseu* – Pseudomonadales, *Rhiz* –

Rhizobiales, *Rhod* – *Rhodospirillales*, *Sapr* – *Saprospirales*, *Sphi* – *Sphingobacteriales*. Phyla: *A* – *Actinobacteria*, *B* – *Bacteroidetes*, *F* – *Firmicutes*, *Fu* – *Fusobacteria*, *P* – *Proteobacteria* (classes *aP* – *Alphaproteobacteria*, *bP* – *Betaproteobacteria*, *gP* – *Gammaproteobacteria*). BAL – bronchoalveolar lavage fluid; LUNG.DP – non-malignant distal piece, LUNG.PT – peritumoural tissue; LUNG.T – tumour. N – neutrophils. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$

3 Discussion

Even though lung cancer is the leading cause of death from cancer worldwide [1], its connection to lung microbiota and the interaction of lung microbiota and lung cancer immunology remains largely unexplored. This is, to our knowledge, the first study in NSCLC analysing lung microbiota between stages based on metastatic LN. Moreover, lung microbiota was analysed for the first time in four different samples: BAL as a sample of alveolar “planktonic” microbiota, and three tissue samples harbouring different physiology: tumour, peritumoural tissue (tissue in direct contact with tumour and with increasing importance in deciding tumour progression) [36,37] and non-malignant tissue. In addition, in our study, BAL was not obtained by bronchoscopy but by direct instillation of physiological serum into the dissected lung, limiting upper airways contamination and giving a possibility of true sampling of the microbiota in tumour proximity.

We showed that each Stage group revealed differences between and within samples’ community compositions (Fig. 1). Beta diversity differed significantly between Stage groups both in tumour and non-malignant tissue. Consequently, peritumoural tissue was compositionally more similar to non-malignant tissue in Stage I,II, and inversely, to tumour and BAL in Stage III. We also observed that the Stage III group had lower alpha diversities in peritumoural and non-malignant tissue compared to Stage I,II, with no change in the other two samples. Lung tissues were dominated by phylum *Proteobacteria* in Stage I,II, but in Stage III, tumour and peritumoural tissue were dominated by *Firmicutes*. Yu et al. [27] also identified *Proteobacteria* as the dominant phylum in non-malignant lung tissue in lung cancer patients. However, in their study, the alpha diversity of non-malignant tissue was higher in more advanced stages, which is the inverse of our finding. This could be because they analysed stages using the base TNM classification [33], which classifies patients into stages depending on the overall TNM score and not exclusively based on metastatic LN as we performed. In our study, BAL samples were dominated by phylum *Firmicutes* without difference relative to Stage group. Interestingly, the study of Lee et al. [25] found abundance of phylum *Firmicutes* increased in BAL of lung cancer patients compared to those with benign mass lesions. In addition, we observed phylum *Firmicutes* as dominant in tumour and peritumoural tissue of Stage III

patients, two samples significantly more similar to BAL in this group. Looking at these findings, the shift towards phylum *Firmicutes* might be associated with advancement of lung cancer.

We identified classes and genera for each lung tissue sample with significant difference in abundance between two tumour stage groups (Fig. 2). Interestingly, in tumour, the 1st ranked class by the importance of difference was class *TM7-3*, more abundant in Stage III. This class is from the candidate phylum *TM7*, previously reported as increased both in COPD and lung cancer [38] and could be implicated in tumour progression. Looking at both analysis on the class and genus level, we noted the consistent inversion of differential abundance profiles between tumour vs. peritumoural/non-malignant tissue. In Stage III, genera more abundant in tumour were less abundant in peritumoural/non-malignant tissue, and it was the inverse in Stage I,II. Interestingly, members in each of these “inversion groups” mostly belonged to the same higher taxonomic levels, but on the contrary, these higher levels were not shared between “inversion groups”.

Next, we divided identified genera into two “abundance change” hypotheses depending on their abundance in tissues and stages. Interestingly, each hypothesis gathered members that belonged to one of the major respiratory profiles. The first hypothesis included aerobic bacteria, more abundant in Stage I, II tumour and scarcely or not present in the other two tissues, and inversely, in Stage III genera more abundant in peritumoural and non-malignant tissue with lower abundance in tumour. The second hypothesis involved anaerobic genera, more abundant in peritumoural and non-malignant tissue in stage I, II. Those in Stage III have increased abundance in tumour, and decreased abundance in the other two tissues compared to Stage I,II. We hypothesise that aerobic bacteria from the first group are more abundant in Stage I,II tumour tissue due to increased protection from the immune system and increased availability of nutrients [39]. Low-grade tumours are still well oxygenated and could sustain aerobic bacteria. However, with stage advancing and/or tumour growth, the supply of oxygen and nutrients becomes insufficient for tumour cells, inducing hypoxia and necrosis within the tumour centre, accompanied by increased lactate production and acidification [40,41]. The aerobic bacteria that are not able to live in these conditions “leave” the tumour, i.e. move to peritumoural and non-malignant tissue. Even though only ¼ of detected genera (Fig. 3) had active motility organs (flagella), the literature suggests another means of motility, such as “gliding” or attachment on eukaryotic cells [42,43] that could support this “migration”. In this study, we analysed only a captured moment and we have no insight into microbial evolution and changes following

development of the tumour. However, in multiple examples genera were still completely absent in one group (0%) but present in the other (Fig. 3). Rather than only changing a balance between microbiota proportions (saying that microbiota already previously existed in the tissues before the change in abundance), our results of beta diversity on uwUF (based on presence/absence of OTUs) support the hypothesis that certain bacteria might “migrate” between tissues. Also, in certain genera, peritumoural tissue has much smaller oscillations between two Stage groups compared to non-malignant tissue and tumour, which could be due to its position in the middle of this “migratory” pathway. For the second group, we suggest that anaerobic bacteria could reside inside the biofilms in the non-malignant and peritumoural tissue, which protects them from toxic oxygen exposure. The suggestion of biofilm importance in lung microbial co-location of aerobes and anaerobes, as well as in pathogenesis of lung diseases was previously suggested [15,44–46]. The majority of anaerobic bacteria from the second hypothesis ferment glucose to produce lactate [47,48], but also other acids such as butyric, acetic or propionic (e.g. *Faecalibacterium*, *Odoribacter*, *Ruminococcus*, *Bifidobacterium*) [49,50]. Since tumour cells are proposed to influence glucose delivery of the host to the tumour bed [51], this could be the true chemotactic target of both aerobic and anaerobic bacteria. Once tumour environment becomes sufficiently hypoxic, anaerobic bacteria could “migrate” from the surrounding biofilm into the tumour bed. The tumour-targeting anaerobic bacteria were already described in the context of bacteria-mediated tumour therapy (BMTT). Innate principles of BMTT are based either on stimulation of CD8⁺ T cells through bacterial epitopes, resulting in enhanced tumour surveillance (seen in anaerobic *Clostridium* spores), or on direct antitumour effect through nutrient competition with tumour cells (e.g. strains of *Clostridium*, *Bifidobacterium* and *Salmonella*) [39]. On the other hand, tumour acidification and hypoxia impair activation and anti-tumour response of immune cells [52], and, as mentioned, anaerobic bacteria contribute to lactic acid production and acidification. This hypothesis corresponds to our finding, where anaerobic microbiota was enriched within the tumour of more advanced stages characterised by metastatic nodes.

From genera in Fig. 3 we identified several potential biomarkers (Fig. 4) that, interestingly, all belonged to anaerobic bacteria from the second abundance change hypothesis. Moreover, all except *Parabacteroides* belonged to order *Clostridiales* from the phylum *Firmicutes*. In each tissue, the AUC percentage of combined genera was high, showing that, potentially, any of the tissues could serve as an indicator of metastatic LN presence, improve disease follow-up, and

could limit the necessity of node sampling and analysis. However, selected genera need confirmation of quantitative analysis (such as qPCR) and larger study population.

Looking at local immune profiles (TILs, BAL immune cells), the only observed difference was in percentage of Th17 cells, significantly elevated in BAL of the Stage III group. Consequently, the Treg/Th17 ratio, to which high importance in lung cancer immunity was recently attributed [53], was lower. Even though certain studies reported Th17 cytokines to affect effector cytotoxic T cell generation and thus stimulate anti-tumour response [54,55], a meta-study analysing the results of 6 lung cancer cohorts (total no. of participants = 479) sided elevated Th17 cytokines with significantly reduced overall and disease free survival, respectively [56]. In our study, the parallel could be drawn with higher levels found in the Stage III group characterised by metastatic lymph nodes as a marker of elevated tumour aggressiveness.

In this study, we exposed first results on relation of different lung microbiota with TILs and immune cells in BAL. Assessment of the association between four lung microbiota with both TILs and immune cells in BAL showed surprisingly similar results. In both analyses, only microbiota from BAL showed clear correlation profiles for multiple markers and correlating genera mutually corresponded between the two analyses. Genera from phyla *Firmicutes* (especially order *Clostridiales*), *Actinobacteria* and *Bacteroidetes* (class *Bacteroidia*) showed positive correlation with anti-tumour markers (TILs: CD8⁺ T lymphocytes, CD20⁺ B lymphocytes, FOXP3⁺ Tregs; in BAL Th1, Th2, Th1/Treg, Th1/Th17, Th1/Th2 ratios and inactive forms of neutrophils) [35]. Several genera in this group were already described for their potential in stimulating antitumoural immunity, such as *Akkermansia*, *Bifidobacterium*, *Lactobacillus* [57,58]. On the contrary, genera from BAL that were positively correlated with an immunosuppressive profile, particularly with BAL immune cells (Treg, Th17, Th17/Treg, activated neutrophils) [35] belonged mostly to phyla *Proteobacteria* (classes *Alphaproteobacteria* and *Gammaproteobacteria*) and *Bacteroidetes* (classes *Flavobacteriia*, *Sphingobacteriia*, *Saprospirae*). Conversely to the first group, genera from this group such as *Haemophilus*, *Streptococcus*, *Staphylococcus*, *Pseudomonas* were previously associated with lung pathologies (as COPD) and negative or immunosuppressive impact on the host [10,14].

Interestingly, many BAL genera that positively correlated with anti-tumour markers in BAL belonged to the same taxonomic lineage as the group of anaerobic genera with increased abundance in Stage III tumours (therefore decreased in non-malignant and peritumoural tissue), and *vice versa* for negatively-correlated genera. Although representing a sample of

inter-bronchial microbiota, BAL genera were significantly associated both with immune markers within tumour and in BAL. Surprisingly, however, microbiota of the tissues, including tumour, showed poor association with immune characteristics from both TILs counts and BAL immune profiles. Further research will be necessary to determine the exact reason for this, since the answer is certainly very complex interplay between microbial, immunological and tumour properties.

The major limitation of this study is the small number of patients due to the strict inclusion criteria. In this study design, we were not able to perform a longitudinal study following tumour progression and co-occurring microbiota modification. It would also be interesting to look at the absolute numbers of both immune cells and specific bacteria to complete these quality based data. This will be a perspective for future research.

4 Conclusion

To our knowledge, this is the first study in NSCLC patients that analysed lung microbiota from four different samples (BAL, non-malignant tissue, peritumoural tissue and tumour) and their relation with local immune response depending on the presence of metastatic lymph nodes. We showed that BAL microbiota was that most associated with both intra and extra-tumour immune response, while lung tissues' microbiota changed relative to presence of metastatic lymph nodes. We also identified several biomarkers in lung tissues that could help improve patient's diagnosis and prognosis. These findings need further confirmation in larger cohorts and additional research on interaction of various lung microbiota and lung cancer with its potential use in diagnostics.

5 Materials and methods

5.1 Patient recruitment

Patients pre-considered for inclusion in the study were patients diagnosed with primary NSCLC eligible for surgical treatment without neoadjuvant platinum-based chemotherapy and presented before the Thoracic Oncologic Committee of the Centre Jean Perrin, Clermont-Ferrand, France. Inclusion criteria were: age between 18 and 80 years, body mass index (BMI) < 29.9, no administration of antibiotics, corticosteroids, and immunosuppressive drugs or no history of lung infection in at least the past 2 months as well as no previous airways surgery/cancer treatment. Detailed inclusion/exclusion criteria are available elsewhere [34]. Each patient was attributed a number at inclusion that was used for subsequent analyses.

5.2 Study design

The study was concentrated around two points: inclusion and surgery. During an outpatient appointment preceding surgical intervention, eligible patients gave their written informed consent to participate in the study. The sampling of blood and lung tissues and fluid was done the day before surgery and during surgery, respectively. Lung sampling was performed on excised lobe during the operation and, therefore, posed no additional inconvenience for the patient. Four different lung samples were chosen as each represents a distinct microbiota relative to the lung physiology of the sample's origin. A study flowchart as well as detailed design and power calculation were previously published [34].

5.3 Sampling of lung tissue and BAL

The detailed procedure was previously published elsewhere [34]. Briefly, immediately after partial or complete pneumonectomy, the lobe was washed by instillation of sterile physiological serum (2 x 40 mL) directly to the bronchus to obtain BAL fluid (retrieved 8-10 mL in total). Furthermore, a non-malignant piece distal to the tumour was excised from the same lobe and designated as LUNG.DP. Tumour and peritumoural tissue were excised together as a pie-slice, to sample both the surface and centre of the tumour. Carefully, peritumoural tissue was cut off from the tumour slice based on its histological qualities. The tumour tissue sample was designated as LUNG.T and the peritumoural tissue sample as LUNG.PT. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction. 3 mL of BAL were used for immune cell phenotyping, while the rest was directly stored at -80°C until DNA extraction.

5.4 Tumour immune infiltrate

Immunohistochemistry was performed on deparaffinised tumour tissue using the specific antibodies to detect subpopulations of immune cells as follows: cytotoxic T-lymphocytes (anti-CD8, clone SP16, ThermoFisher Scientific, dilution 1:200), regulatory T-lymphocytes (anti-FOXP3, clone SP97, ThermoFisher Scientific, dilution 1:100), B-lymphocytes (anti-CD20, clone SP32, Cell Marque, dilution 1:100). The immune response checkpoint axis PD-1–PD-L1 was assessed by anti-PD-1 (clone NAT105, CellMarque, dilution 1:50) and anti-PD-L1 (clone 28-8, Abcam, dilution 1:50). All staining was performed by a fully automated, standardised procedure (Benchmark XT, Ventana/Roche). The number of lymphoid cells expressing each antigen, except PD-L1, was determined on five consecutive high-pass filter (HPF) (×400) fields (Nikon Eclipse Ci microscope), starting from the invasive front toward the

tumour centre as previously described [59]. It was used as a parameter reflecting the tumour's quantity of a given immune cell subpopulation. The five-field average was used for statistical analysis. PD-L1 was assessed for both immune and tumour cells and reported as the percentage of each population expressing the antigen.

5.5 BAL immune cell phenotyping

Leukocytes were obtained after haemolysis from fresh blood and BAL as previously described [34]. Leukocytes were phenotyped by flow cytometry (LSRII, BD Biosciences) with the following antibodies: anti-CD3-VioBlue, anti-CD4-APC-Vio770, anti-CD25-APC, anti-CD127-VioBright FITC, anti-CD183 (CXCR3)-PE-Vio770, anti-CD294 (CRTH2)- PE, anti-CD196 (CCR6)-PE-Vio615, anti-CD15-FITC, anti-CD62L-PE, anti-CD11b-PE-Vio770 and Viability 405/520 fixable dye, all purchased from Miltenyi Biotec. Antibodies were used in dilution 1:10 and viability dye in 1:50.

Granulocyte and lymphocyte population was obtained as a percentage of overall leukocytes gated on SSC/FSC plot. In the lymphocyte population, CD3⁺CD4⁺ helper T lymphocytes subpopulation was characterised as follows: Th1 as CXCR3⁺, Th2 as CRTH2⁺, Th17 as CCR6⁺ and Treg as CD25⁺CD127⁻. In the granulocyte population, neutrophil subpopulations were characterised as follows: circulating neutrophils as CD15⁺CD62L⁺CD11b⁻, transient “tethering” form as CD15⁺CD62L⁺CD11b⁺, and activated neutrophils as CD15⁺CD62L⁻CD11b⁺. Neutrophil subtypes are presented as percentage of CD15⁺ granulocytes. Both lymphocyte and neutrophil gating strategy is shown in Additional file 1: Figure 2. Compensations and controls used the FMO (Fluorescence Minus One) procedure with corresponding antibody isotypes. The phenotyping of Th lymphocytes was based on previously published gating strategy but without viability marker [60]. The program used was Kaluza Analysis 2.1 (Beckman Coulter).

5.6 DNA extraction and negative controls

DNA was extracted as previously described [34]. Lung tissue samples (three samples per patient) were randomised so that as each extraction group never contained only one sample type or all samples from the same patient to alleviate the bias of manipulation. All samples were extracted in two batches.

Since the study involves low biomass samples, background controls were performed throughout the sampling and extraction procedure. The control sample of physiological serum used for

BAL was taken from the same vessel and with the same syringe used for lavage afterwards. During the DNA extraction, miliQ water was used as a negative background control, treated with all the reagents and passing all the procedures along with the real samples. All controls were sequenced and analysed along with the samples. All the reagents used in DNA extraction and sample pre-treatments were either autoclaved, filtered through 20 µm filters or purchased sterile. All the tools and pipettes were thoroughly washed and disinfected between extractions of different sample types or between different extraction steps.

5.7 16S ribosomal RNA (16S rRNA) gene sequencing

16S ribosomal RNA gene sequencing was performed by DNA Vision, Belgium, using Illumina MiSeq technology. After PCR amplification of the targeted region V3-V4, libraries were indexed using the NEXTERA XT Index kit V2. The sequencing was carried out in paired-end sequencing (2 x 250 bp) by targeting an average of 10,000 reads per sample. Sample sequences were clustered to OTUs based on 97% sequence similarity. The software used was QIIME (Quantitative Insights Into Microbial Ecology). The generated “raw” OTU table, taxonomy and Newick formatted phylogenetic tree provided by DNA Vision were used for further bioinformatics analyses.

5.8 Sequence processing and microbiota analysis

Microbiota statistical analysis and visualisation were performed using RStudio [61] (packages “phyloseq” [62], “vegan” [63], “microbiome” [64], “microbiomeSeq” [65], “ggplot2” [66], and “pROC” [67]).

The “raw” OTU table obtained from DNA Vision contained two kingdoms, *Bacteria* and *Archaea*. Only *Bacteria* were kept for further analysis. Furthermore, the OTU counts of each BAL sampling control (physiological serum taken at the sampling of BAL) were subtracted from its corresponding sample. Likewise, the OTU counts of each or multiple extraction controls were subtracted from samples corresponding to extraction. Mean read no. of 26 negative controls was 86, with average of 21 OTUs detected per control, which gives an average of 4 reads/detected OTU. None of these OTUs belonged to more represented OTUs in true samples, and had mostly lower counts than in negative controls. Since samples were extracted in two batches (with complete randomisation of all sample types), OTUs with 0 count in each sample of one batch and detected in each sample of the other batch were excluded from further analysis. Detected OTUs represented marginal count values and left virtually unchanged data. Only OTUs present in at least 10% of the samples or having more than 50 overall counts were

kept for further processing (in our case, this was equivalent to keeping the OTUs with minimal average abundance of 0.001%). Only samples with more than 1,000 reads were included in the analysis. This excluded 6 samples: in the Stage I,II group 1 BAL, 2 peritumoural tissues and 1 tumour, and in the Stage III group 1 BAL and 1 peritumoural tissue. Average read number of final sample groups was $17,046 \pm 14,879$ (mean \pm SD) for BAL, $13,352 \pm 12,909$ for non-malignant tissue, $13,039 \pm 11,394$ for peritumoural tissue and $5,846 \pm 4,505$ for tumour.

For diversity analyses, samples were rarefied at 1,195 reads (min. read no. of samples) with 100 iterations. Alpha diversity was estimated by observed OTU number, Shannon diversity and Faith's phylogenetic diversity. Analysis of beta diversity (compositional distance between samples or groups of samples) was performed on weighted (more importance on difference in abundance) or unweighted (importance of presence/absence of OTUs) UniFrac distances using Adonis function ("vegan") with 999 permutations and presented with 2D non-metric multidimensional scaling (NMDS).

Relative abundance was calculated from the unrarefied OTU table. Differential abundance between tumour stages for each sample type was determined with the adapted Deseq procedure ("microbiomeSeq") using modelling of abundances as negative binomial distribution (mean depends on sample specific size factor and concentration of given OTU in a sample). This procedure starts with the non-rarefied OTU table, followed by log-relative transformation and Wald's test to test significance of coefficients in a negative binomial general linearized model based on sample estimates. Additional output variables of this procedure is a rank of importance of the detected significant difference, as detected by random forest classifier. Significance threshold was 0.05 after Benjamini-Hochberg (BH) correction.

Taxa detected as significantly different between tumour stages within respective samples were next compared between samples (inside the same stage) by non-paired Kruskal-Wallis test with BH correction.

Receiver operating characteristic (ROC) analysis was done on normalised OTU counts with general linear model fit. pROC package was used for AUC calculation and plot [67].

5.9 Statistical methods for non-microbial or mixed analysis

All statistical analyses and graphics were done using RStudio [61] (package "ggpubr" [68]). The tests used were nonparametric (Man-Whitney, Kruskal-Wallis) and 2-sided with the significance threshold at 0.05 with BH correction for multiple comparison (if not specified

otherwise). Data are shown as boxplots (aligned or dispersed points) with the central line representing median, box ends as first and third quartiles, and error bars as minimum and maximum (higher and lower non-outlier observations) if not specified otherwise. The Chi-square test was used to examine balance of factors such as smoking, sex, tumour type etc. between two Stage groups.

Spearman's correlation was used to determine the association between microbial taxa and environmental variables. Relative transformation of the OTU table was done prior to analysis and BH correction was applied both for grouping variables and taxa. The significance threshold was 0.05.

5.10 Managing missing data – paired/unpaired tests

Due to an insufficient number of reads, 6 lung samples were excluded from the analysis, leaving 62 lung samples overall. Since the excluded samples belonged to different patients and different tissues (no pattern), keeping a strictly paired analysis approach would drastically impact the group size. Therefore, the analyses comparing different lung tissue within the same tumour stage were unpaired.

In only one patient (Stage I,II) immune cells in BAL were not phenotyped. Patient was therefore excluded only from the paired analysis that included BAL immune profiling and association with BAL microbiota.

6 Declarations

6.1 Ethics approval and consent to participate

This study (and its amendment in June 2018) was approved by the Ethics Committee (Comité de protection des personnes (CPP) Sud-Est VI), Clermont-Ferrand, France, and the French National Agency for Medicines and Health Products Safety (ANSM) (study ref. 2016-A01640-51). The study was registered with the Clinical Trials (clinicaltrials.gov) under ID: NCT03068663. Because of the invasiveness of the sampling techniques, the requested control group was not approved by the CPP. Written informed consent was obtained from all patients upon inclusion in the study.

6.2 Consent for publication

Not applicable.

6.3 Availability of data and material

According to the practice of the sponsoring institution (Centre Jean Perrin), all samples will be preserved for 15 years. The datasets generated and/or analysed during the current study are confidential but are available from the corresponding author on reasonable request and after the data sharing process is brought into compliance with GDPR (Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016).

6.3.1 Data sharing

This statement is intended to clarify Centre Jean Perrin's position on data sharing with regards to applicable regulatory frameworks.

6.3.1.1 European regulatory framework

De-identified study data are considered as personal data within the meaning of Regulation (EU) 2016/679 of the European Parliament and of the Council of 27 April 2016, known as « GDPR ».

As a result, their transfer places further processors in the scope of GDPR (Art. 2 and 3) and is notably subject to

- precise information of study subjects (Art. 14),
- prior identification of further processors and signature of a transfer agreement (Chapter V),
- data protection impact assessment (Art. 35).

6.3.1.2 French regulatory framework

In addition to GDPR, the transfer of this data by Centre Jean Perrin is a processing in itself that is subject to Chapter IX and XII of the updated Law n°78-17 of 6 January 1978. Within the framework of this law, the data processing is permitted by Centre Jean Perrin's conformity to the MR-001 Code of conduct (Délibération n° 2018-153 du 3 mai 2018 portant homologation d'une méthodologie de référence relative aux traitements de données à caractère personnel mis en œuvre dans le cadre des recherches dans le domaine de la santé avec recueil du consentement de la personne concernée).

In its last update from May 2018, MR-001 was updated to allow transfer to reviewers within certain conditions (Article 2.4).

These conditions can be summarised as follow:

The access to study data by an **independent mandated reviewer** can only be granted through an interface chosen by the data processor (i.e. Centre Jean Perrin, in this context) for the sole purpose of re-analysis or for any other purpose supported by a strong rationale and a project synopsis describing why access to the this study's data (study acronym: MICA) is necessary.

The data processor shall ensure:

- That the chosen sharing platform cannot allow extraction of the data;
- To grant specific personal and differentiated authorizations to access the data;
- That the users be reliably identified
- That state of the art cryptography and security measures are used;
- That an audit trail of accesses is used;
- When data is transferred outside of the EU, that applicable transfer contracts are established;
- That study subjects are informed on the data recipients;
- That the data is used for the sole purpose of reproducing published results;
- That the data is cleaned from any directly identifying data and that the principle of data minimization is applied (i.e. limiting the transfer to data used in the publication).

6.3.1.3 Study Data sharing

As a result, for this trial (acronym: MICA), Centre Jean Perrin can share individual participant data that underlie the results reported in the article under the following conditions:

- Limitation to the data processing to the purpose of independent review of the published results;
- Prior minimization of the data (i.e. restriction to data strictly adequate, relevant and limited to the purpose of the independent review of results);
- Prior identification of the reviewer and authorization from Centre Jean Perrin for a personal access;
- Following the signature of a transfer agreement with Centre Jean Perrin, in conformity with the European Commission's Standard Contractual Clauses;

Access to study data will be assessed, upon written detailed request sent to the project manager Emilie THIVAT: emilie.thivat@clermont.unicancer.fr, from 6 months until 5 years following article's publication.

6.4 Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6.5 Funding

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6.6 Authors' contributions

ABD, EF, JYB, MF and MPV contributed to the study design. ABD, EF, MF, NRR, and RB planned the experiments. JT, MF, NRR and RB, performed the experiments. Both NRR and RB analysed immunohistochemical data, and RB analysed remaining data. ABD, MF, NRR and RB conceived the idea here presented. ABD, JT, MF and NRR helped in interpreting the results. RB designed the figures and wrote the manuscript. All authors gave their critical feedback for the final manuscript version.

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8 Additional files

Additional file 1.pdf – contains two figures:

- Figure 1. TILs ratio in tumour stages and correlation with lung microbiota. – Additional figure showing TILs ratio relative to Stage group (Fig. 1a), and its association to lung microbiota (Fig. 1b).
- Figure 2. Gating strategy for phenotyping of T helper lymphocytes and granulocytic neutrophils in bronchoalveolar lavage fluid. – additional figure illustrating the gating strategy on the representative BAL sample.

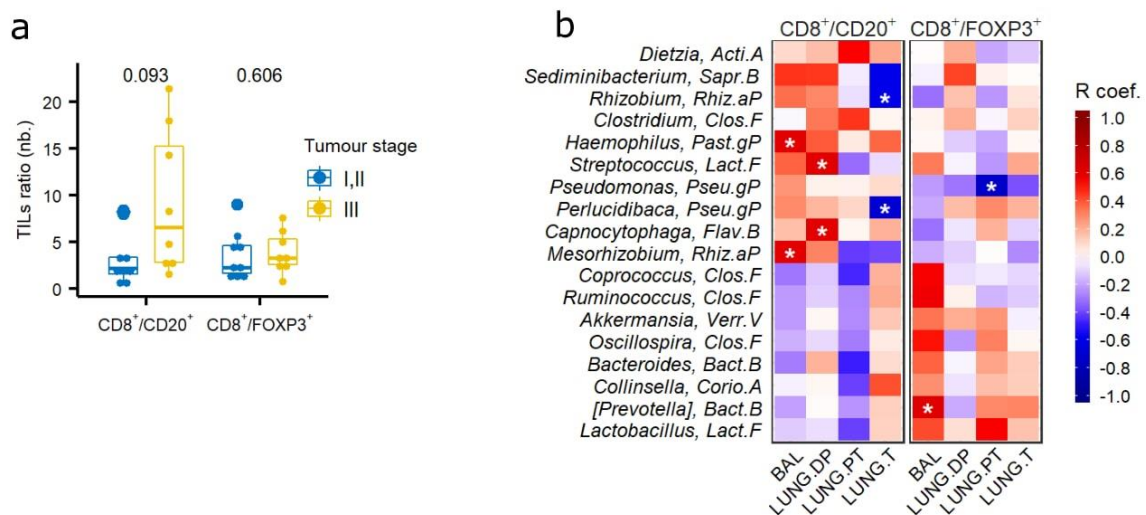


Figure 1. TILs in tumour stages and correlation with lung microbiota. **a** Difference in TILs ratios between tumour stages. **b** Spearman's correlation between microbiota of four lung samples with two TILs ratios. Colour represents correlation coefficient. Shown significance levels are adjusted p-values based on BH correction. Only genera with at least one significant entry are presented. Sample numbers for correlation were: LUNG.T (N = 16), LUNG.PT (N = 14), LUNG.DP (N = 17), BAL (N = 15). TILs – tumour infiltrated lymphocytes. Following full taxa names are corresponding order (4 letters) and phylum (1 capital letter). Orders: *Acti* – Actinomycetales, *Bact* – Bacteroidales, *Clos* – Clostridiales, *Corio* – Coriobacteriales, *Flav* – Flavobacteriales, *Lact* – Lactobacillales, *Past* – Pasteurellales, *Pseu* – Pseudomonadales, *Rhiz* – Rhizobiales, *Sapr* – Saprospirales, *Verr* – Verrucomicrobiales. Phyla: *A* – Actinobacteria, *B* – Bacteroidetes, *F* – Firmicutes, *P* – Proteobacteria (classes *aP* – Alphaproteobacteria, *gP* – Gammaproteobacteria), *V* – Verrucomicrobia. *[Prevotella]* belongs to the family *Paraprevotellaceae*. *: $p < 0.05$

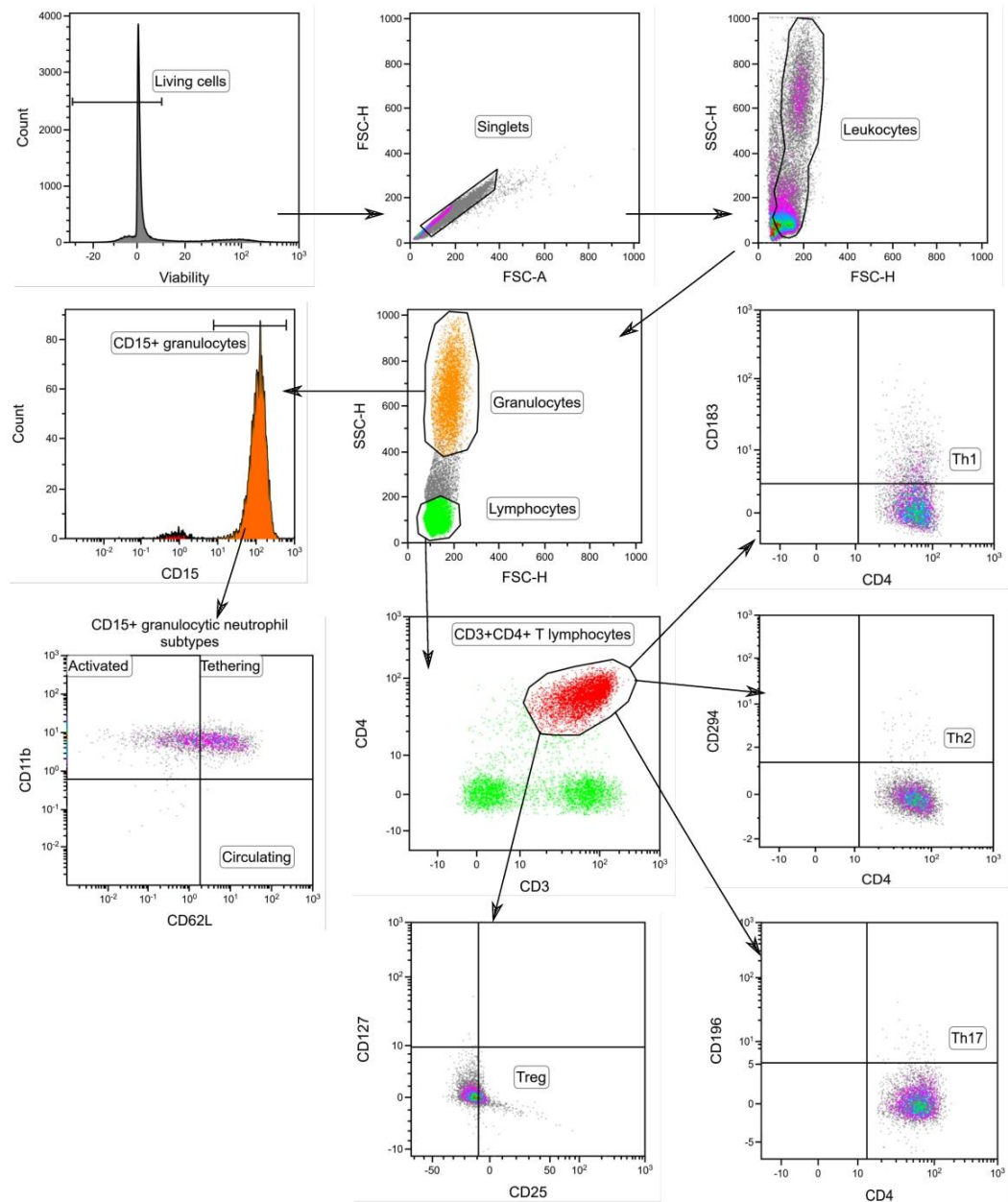


Figure 2. Gating strategy for phenotyping of T helper lymphocytes and granulocytic neutrophils in bronchoalveolar lavage fluid. T helper (Th) cells were identified as $CD3^+CD4^+$ lymphocytes, with further subsets as follows: Th1 cells as $CD4^+CD183^+$, Th2 cells as $CD4^+CD294^+$, Th17 cells as $CD4^+CD196^+$, and Treg cells as $CD25^+CD127^-$. Granulocytic neutrophils were subtyped from granulocytes population as follows: $CD15^+CD62L^+CD11b^-$ as circulating inactive neutrophils, $CD15^+CD62L^+CD11b^+$ as tethering neutrophils, $CD15^+CD62L^-CD11b^+$ as activated neutrophils.

General discussion, conclusion and perspectives

1 Discussion

In the history of the research, we could say that the progress of one field was never linear and different scientific branches never developed simultaneously. One crucial advancement in the area opens the door into a new and unexplored world, with numerous possibilities and offering answers to all the problems. The scientific community has already been marked with the decades of genomics, proteomics, metabolomics, but every next questioned the “divinity” of the previous. At some point however, there were researchers who were capable to see beyond the “mainstream” movement, trying to put the things in perspective, beginning to understand the organism is a puzzle requiring all its pieces in the right place to maintain its homeostasis. This encouraged the new approach in the research, which then started to use all the developed tools to fill in the missing pieces and enable seeing the final picture. In this context began the studies of the microbiota, starting to connect the intestinal homeostasis or dysbiosis with host’s wellbeing, biological paraphrase of “we are what we eat”. Even though the last decade could definitely be called the decade of the microbiota, this new “divinity” was well intertwined with other crucial domains, as immunity, physiology and genetics. The interaction of the gut microbiota with local mucosa, mesenteric lymph nodes and successively, distal immune disorders, opened a vast field for research, discussion and potential application of bacteria in the treatment of the major modern world problem, cancer (Daillère et al. 2016; Sivan et al. 2015; Vétizou et al. 2015; Viaud et al. 2013). Lung cancer, with leading incidence and mortality worldwide (International Agency for Research on Cancer 2019), is definitely recognised as the issue with the heaviest impact on the quality of life but also on the social care system. One of the reasons is its late and mostly accidental discovery in already advanced stages in aged population, which will too often give into the side effects of adjuvant therapies, surgery or tumour itself. Therefore, the stimulation of the anticancer effect or improvement of the therapy by resident microbiota draw the attention of the medical circles. However, as these advancements are based on animal models and are of relatively recent dates, the studies including human subjects remain a few, with additional inconvenience – low knowledge of the effect of the local, in this case, lung microbiota.

Inspired by the idea of the gut-lung axis (discussed in the published review (Bingula et al. 2017)), the purpose of this thesis project was to characterise NSCLC patients through multidirectional approach, which would provide essential and currently missing knowledge

crucial for better understanding of communication between major factors implicated in the axis and later development of interventional research. This included characterisation of the microbiota of the lung, gut and saliva, and its association both with local (intratumoural, extratumoural, intestinal) and systemic (blood) immune response with regard to microbial products (as SCFA) and accompanying nutrition status.

Due to multiple possible research axes in this project, we have first decided to concentrate on the lung region, since it is less explored compared to the gut. We have first focused on the characterisation of different lung microbiota (tumour, peritumoural and non-malignant tissue, and BAL), their relation to salivary microbiota and the impact of the location of the tumour lobe (Article 1). Second, we wanted to address the direct clinical application of our research. So, we have investigated whether there is an interaction between the presence of metastatic lymph nodes (as one of the major prognostic factors for NSCLC), different lung microbiota and local immunity (Article 2). Further research axes will be discussed in Conclusion and perspectives.

❖ **BAL represents unique lung microbiota and is only marginally more similar to saliva than are the lung tissues**

Since its beginnings, the investigation of the lung microbiota has been accompanied by several inconveniences. One of the major remained the choice of the lung sample that would well represent the lung communities but that would not be too invasive for the patient. Therefore, BAL was and still is the most frequent sample type in studies of the lung microbiota, including lung cancer (Laroumagne et al. 2011; Lee et al. 2016; Liu et al. 2018; Tsay et al. 2018; Wang et al. 2019). However, it has been previously suggested that lung microbiota found in BAL and in lung tissues do not share same characteristics (Sze et al. 2012), due to different physiology of samples.

We have shown for the first time that BAL microbiota harbours distinct microbiota compared to lung tissue while including up to three different tissue samples (non-malignant, peritumoural and tumour tissue) (Article 1). BAL represented a unique sample based on both beta diversity and on differential abundance analysis. *Firmicutes* was the most abundant phylum, conversely to *Proteobacteria* in lung tissue samples. Furthermore, looking separately characteristics of microbiota in either upper (UL) or medium/lower lobes (LL), we identified whole clades with inverse abundance between BAL and extratumoural tissue

microbiota, such as *Flavobacteriia* and *Actinobacteria*, confirming the initial premise of two distinct microbial communities.

Since in our study design **BAL was obtained directly on the excised lobe for the first time**, we had the opportunity to estimate if BAL from UL was more similar to saliva (as a representative of upper airways microbiota) than if it was from LL. Following the theory of the adapted island model of the lung biogeography (Dickson et al. 2015), upper lobes should share more similar community composition with upper airways, and show increased richness compared to LL. However, we have not found a significant difference in distance when comparing saliva with BAL from lower or upper tumour lobe, despite slightly increased abundance of typically oral taxa in the UL (as *Veillonella* or *Prevotella*). This could be either due to tumour presence influencing the microbiota properties, as previously suggested (Liu et al. 2018) or because of different sampling approach that importantly lowered the upper airways contamination. On the contrary, both richness and diversity were significantly lower in the LL BAL. Since these did not change in tumour and non-malignant tissue, there are several possible reasons. First that the BAL is more sensitive to distance from the central bronchus than the tissues, causing lower richness in LL according to the island hypothesis (Dickson et al. 2015). Second, since upper lobes of the lung are more expanded than the lower lobes during slow inspiration, more inspired air will enter the lower lobes than the upper lobes (Chang and Yu 1999). This could suggest more dynamic environment in the LL than in the UL, limiting the establishment of richer community, unlike in the upper airways. This will remain the subject for further research.

❖ Peritumoural tissue microbiota as a potential indirect marker of modification of the tumour environment

It has been demonstrated that tumour progression is not only related to tumour itself, but also to its environment. The remodelling of the ECM under tumour-producing factors to enable tumour progression has already been reported (Altinay 2016) (Figure 10), but also the repressive features of the environment on the tumour (Bissell and Hines 2011). However, no study so far examined whether peritumoural tissue harbours microbiota that differs from the one in the tumour or non-malignant tissue in the case of NSCLC.

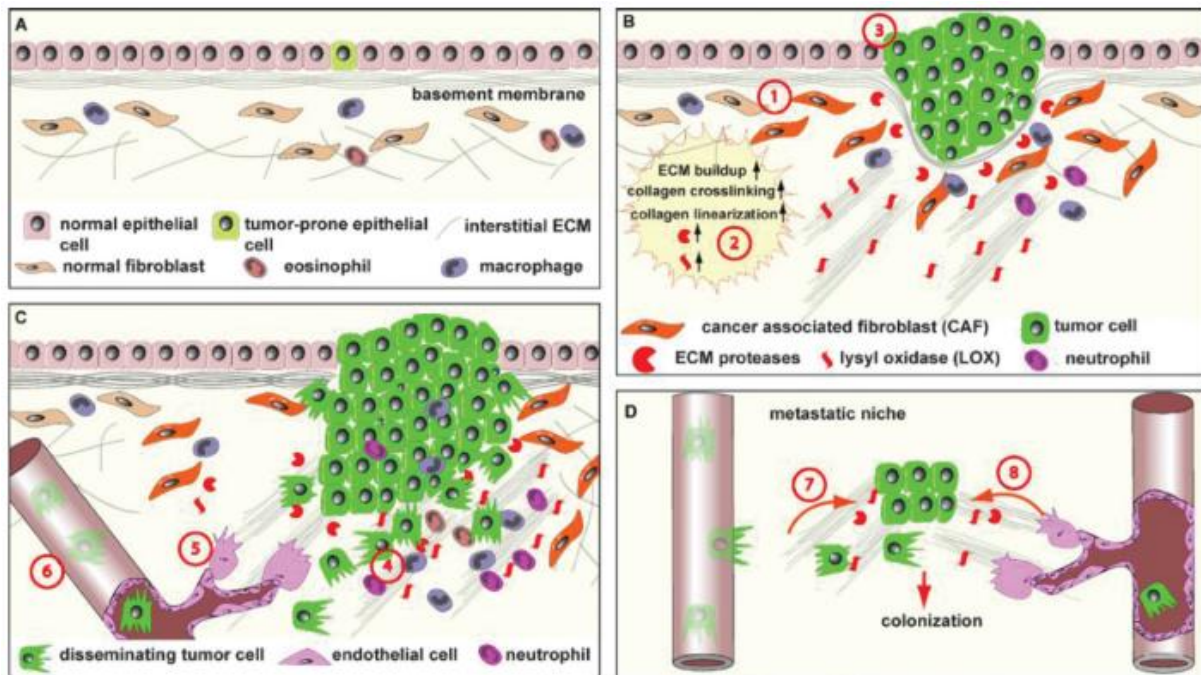


Figure 10 Modification of the extracellular matrix (ECM) promotes cancer progression (adapted from Altinay 2016). (A) normal structure of the extracellular matrix. (B) Under pathological conditions, there is an excessive generation of activated fibroblasts that contribute to overproduction of ECM and deregulation of ECM modelling enzymes. Due to the delicate equilibrium between the local cells and ECM, these changes stimulate epithelial cellular transformation and hyperplasia, creating a stiffened matrix. (C) In addition to local cells, immune cells recruited to the tumour site further promote local inflammation, accompanied by neoangiogenesis facilitating tumour propagation due to the leaky tumour vasculature. (D) Metastases that survived in the circulation, extravasate at distant site and express ECM remodelling enzymes to create a local metastatic niche that will furtherly promote survival and proliferation of cancer cells.

We are the first to include peritumoural tissue in characterisation of the lung microbiota (Article 1), showing that it is the sole lung tissue sample with significant changes in richness and in alpha and beta diversity relative to the lobe location. We reported that peritumoural tissue from UL had increased similarity to BAL (seen in beta diversity and its higher portion of phylum *Firmicutes*) while in LL it demonstrated same features as other two tissues. We proposed that this could actually reflect different type of modifications of the ECM, influencing both adhesive properties and the tissue's architecture (Altinay 2016; Bissell and Hines 2011; Quail and Joyce 2013) and directly modifying local conditions for bacterial growth. In the pathogenicity of NSCLC, tumours from lower lobes have been associated with decreased overall survival, but still without clear explanation (Kudo et al. 2012; Riquet et al. 2016). Our discovery could indicate the indirect detection of subtle differences in the tumour environment that could be connected either to its increased aggressiveness, or these differences could enable

growth of different types of microbiota that might enable or disable cancer progression (e.g. occupying adhesive sites and enabling metastatic progression).

❖ Lower lobes and presence of metastatic lymph nodes marked by higher abundance of the phylum *Firmicutes*

Several studies have already reported the increase of phylum *Firmicutes* in the lung microbiota when comparing control group to the malignant one. This observation has been of especial interest in COPD (Sze et al. 2012) since studies have shown that COPD could lead to development of the lung cancer but also that both diseases are based on clinical or subclinical inflammation creating the mutual “reasoning” ground (Houghton 2013; Melkamu et al. 2013).

We are the **first to report that the lung microbiota based on four different sample types had higher average abundance of the phylum *Firmicutes* if the tumour was found in the lower lung lobes (Article 1)**. Moreover, we noted the increase of *Firmicutes* in lung tissues if **the patient was diagnosed with metastatic changes in the local lymph nodes (Article 2)**. Both of these results are in concordance with the previous findings connecting this shift with advanced disease state or its presence as mentioned in the previous paragraph. In our study, this “shift” was mostly due to significantly higher relative abundance of the genus *Clostridium*. However, the more precise determination of involved species as well as the absolute quantity and the implication in tumour immunology remains the subject for the future research.

❖ Metastatic lymph nodes stratify tissue microbiota as tumour versus extratumoural tissues in an inverse pattern corresponding to bacterial respiratory profiles

Tumour staging is the main prognostic tool in NSCLC and serves in the choice of the appropriate treatment (Goldstraw et al. 2016). It is based on TNM classification that includes the information of patient’s tumour size, involvement of the metastatic changes on the ipsilateral and mediastinal lymph nodes (LN), and occurrence of distal metastasis. The presence of metastatic changes drastically worsens the prognosis compared to e.g. patients with larger tumours but without metastatic changes, since their presence has been associated to shorter 5-year and overall survival (Goldstraw et al. 2016; Planchard et al. 2019).

We are the first to show that the tissue lung microbiota shows specific and inverse pattern between tumour and extratumoural tissues depending on the lymph node metastatic status (Article 2). Moreover, we have showed that **this pattern was closely related to bacterial respiratory profiles**. As the advantage over previous studies, we had three different

types of the lung tissue, non-malignant, peritumoural and tumour tissue, and therefore the possibility to better characterise the microbial communities within and in the immediate and general vicinity of the tumour. 31 genera had significantly different abundance in at least one of the tissues between patients with and without LN metastasis. Curiously, genera appearing in non-malignant and peritumoural tissue seemed to have the opposite abundance pattern from the tumour. It means that if one genus detected in extratumoural tissues was significantly more abundant in patients with metastatic LN than in the ones without, this same genus showed exactly the opposite if it was detected in the tumour (Article 2, Figures 2 and 3). Although not all 31 genera had significantly inverse abundance for each tissue, they all showed the same tendency to follow this general pattern. Even more striking was the fact that genera with higher abundance in the tumours from patients with normal LN and in extratumoural tissues from patients with metastatic LN all had aerobic respiratory profile and belonged mostly to phylum *Proteobacteria*. Conversely, the genera with the inverse pattern had anaerobic respiratory profile and belonged in the majority to the phylum *Firmicutes*. The inverse abundance distinct respiratory profiles and less changing abundance in the peritumoural tissue led to the hypothesis of the “migratory” bacteria. Looking into the tumour physiology, smaller and non-aggressive tumours are often well oxygenated, with sufficient blood and nutrient supply while providing increased protection from the immune system due to decreased immunogenicity or immunosuppression (Van Dessel et al. 2015). Tumour cells can also produce different factors that centre glucose delivery to tumour cells (Schwartzburd 2019). These conditions could provide excellent environment for aerobic bacteria. However, with tumour development and growth, there is an increased rate of lactic acid production due to its anaerobic metabolism, growing hypoxic conditions and insufficient blood supply by defect neoangiogenic vessels (Fadaka et al. 2017; Wouters et al. 2003). This environment becomes hostile for aerobic bacteria that “move out” of the tumour to its surroundings. On the other hand, anaerobic bacteria, previously suggested to inhabit biofilms within the lungs (Costerton et al. 1994; J. W. Costerton, Stewart, and Greenberg 1999; Mur et al. 2018; Whitchurch et al. 2002), are attracted by this changing environment, and “move inside” the tumour. Here, they could feed, among other, on necrotic tumour cells, while being protected from the immune system by acidic pH inhibiting immune activity (Damgaci et al. 2018). Even though only 25% of detected genera had active motility organs, alternative ways of bacterial taxis have been described previously, including “gliding” or attachment to host’s cells (Mauriello et al. 2010; Youderian 1998). Therefore, it is possible that there is a dynamic exchange between extra and intratumoural environment

depending on the varying growth conditions and that it could be of great importance for our understanding of tumour-microbiota interaction.

The metastatic status of the LN nodes is determined by their direct biopsy, either before surgery by transbronchial echoendoscopy or during surgery. In our study, we have been able to **identify several biomarkers in each lung tissue that could indicate the involvement of the metastatic LN without the direct necessity of their biopsy**. This remains to be confirmed by the qPCR as the more common technique but could, if validated, immensely facilitate the correct diagnosis in NSCLC patients.

❖ **BAL microbiota not sensitive to presence of metastatic lymph nodes, but the most associated to immune phenotypes in BAL and tumour-infiltrating lymphocytes**

It has been previously shown that the increased abundance of supraglottic taxa in BAL correlates with increased inflammatory status in the lungs of lung cancer patients seen by the change in Th17 profile (Segal et al. 2016). However, no study so far evaluated the association between intratumoural immunity and lung microbiota.

We are the first to show that BAL microbiota is associated both with anti and proinflammatory immune profile in the BAL and with tumour infiltrating lymphocytes (TILs) (Article 2). Unlike tissues, BAL microbiota showed no significant difference in the abundance relative to involvement of the metastatic LN. However, this was the only microbiota that showed a significant association with local immunity. We found that members of phylum *Proteobacteria* positively correlated with protumour markers (Chraa et al. 2019) as activated neutrophils, Th17, Treg in BAL and with lower infiltration rate of the tumour. On the other hand, members mostly from the phylum *Firmicutes* positively correlated with the antitumour markers as Th1, Th2, inactive forms of neutrophils (Chraa et al. 2019) in BAL and with higher lymphocyte infiltration rate of the tumour. Several of the detected genera have been previously reported to have similar effect on immune response. E.g. *Akkermansia*, *Bifidobacterium* and *Lactobacillus* were detected as genera with antitumour effect (Bashiardes et al. 2017; Naito, Uchiyama, and Takagi 2018), and *Haemophilus*, *Streptococcus*, *Staphylococcus*, and *Pseudomonas* as associated with lung pathologies (as COPD) and negative or immunosuppressive impact on the host (Erb-Downward et al. 2011; Sze et al. 2015).

2 Conclusion

The lung cancer is the first cause of death by cancer worldwide and the cancer with the highest incidence in 2018 along with the breast cancer (International Agency for Research on Cancer 2019). From two major subtypes, NSCLC takes up to 80-85% of overall cases of lung cancer and hits in majority the older population (Takayuki et al. 2018). While its genetic base is already well characterised (Aisner and Marshall 2012), the interaction between NSCLC and lung microbiota counts its first studies. Even though last decade was marked by the growing interest in the lung microbiota, especially in COPD, asthma and cystic fibrosis (Mao et al. 2018; O'Dwyer, Dickson, and Moore 2016), lung cancer seemed to stay in the shadow of interest. However, this started to change following the recent research in animal models, showing that gut microbiota could influence both tumour growth and host's response to chemotherapy (Iida et al. 2013; Routy, Le Chatelier, et al. 2018; Sivan et al. 2015; Vétizou et al. 2015). These findings evoked the idea of the gut-lung axis, the hypothesis of immune stimulation of the host by certain intestinal commensal bacteria to increase its antitumour activity in the lung (Bingula et al. 2017; Budden et al. 2016; Hauptmann and Schaible 2016; Marsland et al. 2015). Nevertheless, before considering the axis and its distal effect, it was first necessary to investigate the local effect of the lung microbiota (Dickson and Cox 2017). Here, there have been multiple challenges, starting with the ethics of the lung sampling in human subjects focusing the majority of studies to analysis of BAL (Mao et al. 2018). Finally, many issues remained unanswered, from the basic difference between different lung samples and its implication in the studies' conclusions to deeper investigation of tumour-microbiota interaction.

We have shown for the first time that lung microbiota from BAL and lung tissues harbours different characteristics, and that they all represent true lung samples compared to saliva as a sample of oral microbiota. Moreover, we have characterised for the first time the microbiota from four different lung samples, BAL, non-malignant, peritumoural and tumour tissue, and also placing it in perspective of the tumour lobe location. We have shown that the latter influenced the most the microbiota from BAL and peritumoural tissue, while tumour was the least affected. We have also proposed that these changes seen in peritumoural tissue could reflect the modification of tumour's vicinity and influence its aggressiveness.

Next, we have shown that tumour and extratumoural tissue have inverse abundance pattern relative to the involvement of the metastatic lymph nodes. Furthermore, this pattern was closely

related to bacterial respiratory profiles, identifying aerobic bacteria as the more present in tumours without metastatic lymph nodes and in extratumoural tissues with metastatic nodes, and exactly inverse for anaerobic bacteria. We proposed that these two bacterial groups “switch” places relative to the benefit of the tumour microenvironment, as richness in nutrients, protection from the immune system and favourable respiratory conditions. Among these genera we identified potential biomarkers that could add to more accurate and easier clinical diagnosis of lymph node metastases. Finally, we reported the association between local extra and intratumoural immune response and BAL microbiota, identifying members of phylum *Proteobacteria* as associated with protumoural profile and members of phylum *Firmicutes* as associated to antitumoural immune profile. However, this was independent of metastatic lymph node status.

Our findings are among the first that consider lung microbiota in NSCLC and that are significantly adding to the overall understanding of the local dynamics between the microbiota, lung tumour and immunity. Except showing the underlying difference between different lung microbiota, we also demonstrated the dissimilarity in the association with the immune response. Our results will, however, need confirmation in the larger number of subjects, especially for the verification of the potential biomarkers.

3 Perspectives

Even though we have begun to unveil the mystery of the lung microbiota and its implication in the lung cancer, many questions still remain unanswered. As our next objective, we will concentrate to the effect of the metastatic lymph nodes to the rest of the host. This will implicate the analysis of the immune profiles (as already described in BAL) and cytokines in blood, and of course, the investigation of characteristics of faecal microbiota and SCFA as its products for each case. In the scope of the clinical application, we will seek to confirm our discovery of potential biomarkers by developing the absolute quantification with qPCR, to establish the fast and precise technique for detection of metastatic lymph nodes from any tissue biopsy in the tumour lobe.

The immunology of the tumour has become an important factor for consideration when it comes to the choice of the appropriate therapy or prognostics. This is explained by the fact that each tumour belongs to the one of the essential cancer-immune phenotypes, harbouring specific characteristics that influence its interaction with its environment (Chen and Mellman 2017). Since we have observed in our study (Article 2) very heterogeneous counts of TILs and no association to tumour microbiota, it is possible that this might be the discriminating factor. Gut microbiota will also be included in the analysis, since it has been proposed as the initial “stimulator” of the tumour infiltration by anti-tumour immune cells in animal models (Daillère et al. 2016; Routy, Le Chatelier, et al. 2018; Sivan et al. 2015; Viaud et al. 2013).

Regarding bacterial products, we have dosed the SCFA in the faeces, but according to the literature, this represents only 5% of the total produced SCFA in the intestines (den Besten et al. 2013). Even if this is a good indicator of functionary bacterial groups along with the results of enumeration from fresh faeces, it will be interesting to dose SCFA in blood. This would allow us to have insight into the absorbed part, even if it is present in a small amount (Cummings et al. 1987), that might reach the environment of the lung tumour and play the role in immune response as previously suggested (Bingula et al. 2017).

The important perspective of this project is also to complete the group of NSCLC patients that underwent chemotherapy treatment before surgery. This group will enable us to follow the changes in the gut and salivary microbiota and immune markers during the therapy, with surgery as the final point with collection of the lung samples. The chemotherapy side effects due to its non-selectivity, as the disruption of the mucosa and membranes and other fast

proliferating cells, has been one of the major problems in treated population (Yu 2013). However, patients do not react in the same manner, but unfortunately, nor does the tumour, as previously reported (Busch et al. 2016; Catacchio et al. 2018). In animal models, faecal transplantation from responding patients has already been proved as an effective solution (Sivan et al. 2015; Vétizou et al. 2015), indicating that gut microbiota is the one gravely influencing the patient's susceptibility to therapy. Therefore, the objective will also be to try to identify the differences between these patients' subgroups in order to try to influence the non-responders. Considering the lung tumour and lung microbiota, it will be interesting to correlate the degree of tumour response to therapy, as tumour retraction, immune infiltration or degree of necrosis, with both lung and gut microbial factors.

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Annex

Annex 1

Study Protocol Clinical Trial

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Characterisation of gut, lung, and upper airways microbiota in patients with non-small cell lung carcinoma

Study protocol for case-control observational trial

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Abstract

Background: Several studies have confirmed the important role of the gut microbiota in the regulation of immune functions and its correlation with different diseases, including cancer. While brain-gut and liver-gut axes have already been demonstrated, the existence of a lung-gut axis has been suggested more recently, with the idea that changes in the gut microbiota could affect the lung microbiota, and vice versa. Likewise, the close connection between gut microbiota and cancer of proximal sites (intestines, kidneys, liver, etc.) is already well established. However, little is known whether there is a similar relation when looking at world's number one cause of death from cancer—lung cancer.

Objective: Firstly, this study aims to characterise the gut, lung, and upper airways (UAs) microbiota in patients with non-small cell lung cancer (NSCLC) treated with surgery or neoadjuvant chemotherapy plus surgery. Secondly, it aims to evaluate a chemotherapy effect on site-specific microbiota and its influence on immune profile. To our knowledge, this is the 1st study that will analyse multi-site microbiota in NSCLC patients along with site-specific immune response.

Methods: The study is a case-controlled observational trial. Forty NSCLC patients will be divided into 2 groups depending on their anamnesis: Pchir, patients eligible for surgery, or Pct-chir, patients eligible for neoadjuvant chemotherapy plus surgery. Composition of the UAs (saliva), gut (faeces), and lung microbiota (from broncho-alveolar lavage fluid (BALF) and 3 lung pieces: "healthy" tissue distal to tumour, peritumoural tissue and tumour itself) will be analysed in both groups. Immune properties will be evaluated on the local (evaluation of the tumour immune cell infiltrate, tumour classification and properties, immune cell phenotyping in BALF; human neutrophil protein (HNP) 1–3, β -defensin 2, and calprotectin in faeces) and systemic level (blood cytokine and immune cell profile). Short-chain fatty acids (SCFAs) (major products of bacterial fermentation with an effect on immune system) will be dosed in faecal samples. Other factors such as nutrition and smoking status will be recorded for each patient. We hypothesise that smoking status and tumour type/grade will be major factors influencing both microbiota and immune/inflammatory profile of all sampling sites. Furthermore, due to non-selectivity, the same effect is expected from chemotherapy.

Abbreviations: ANSM = The French National Agency for Medicines and Health Products Safety (*Agence nationale de sécurité du médicament et des produits de santé*), BAL = broncho-alveolar lavage, BALF = broncho-alveolar lavage fluid, BMI = body mass index, CIFRE = Industrial Research Training Agreements grant (*Convention industrielle de formation par la recherche*), CRP = C-reactive protein, CT = chemotherapy, ELISA = enzyme-linked immunosorbent assay, FDR = false discovery rate, GF = germ-free, GI = gastrointestinal, HBSS = Hank's balanced salt solution, HNP = human neutrophil peptide, ICI = immune checkpoint inhibitor, IL = interleukin, MCLB = mammalian cell lysis buffer, NSCLC = non-small cell lung cancer, OTU = outer taxonomic unit, Pchir = patient surgery, fr. «patient chirurgie», Pct-chir = patients chemotherapy plus surgery, fr. «patient chimiothérapie — chirurgie», qPCR = quantitative polymerase chain reaction, SCFAs = short-chain fatty acids, UAs = upper airways.

Keywords: chemotherapy, gut-lung axis, immune response, microbiota, non-small cell lung cancer, tumour microenvironment

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1. Introduction

The microbiota is a consortium of different microorganisms that includes bacteria (microbiota), fungi (mycobiota), viruses, and protozoa residing on the skin and in the oral, pulmonary, urogenital and gastrointestinal (GI) cavities, with the GI tract having the highest density of microorganisms. The functional importance of the microbiota to the host is undeniable, involving functions that range from the breakdown of complex dietary polysaccharides to competing with pathogens and modulating the mucosal and immune system in general.^[1] Gut dysbiosis is now considered to be an underlying cause of a wide range of GI diseases and an emerging number of non-GI conditions such as obesity and cardiovascular disease, as well as a range of psychiatric diseases.^[2] Recently, an emerging number of studies began to address the relation between gut microbiota and the lung. This relation has been referred to as the “gut-lung axis”. The basis of this axis theory lies in the “gut-lymph” theory of Samuelson et al^[3] The theory says that the large numbers of macrophages and other immune cells are present in the intestinal submucosa or mesenteric lymph nodes, where the majority of translocating bacteria are also found. If not eliminated by this 1st line defence, surviving bacteria, cell wall fragments or the protein fractions of dead bacteria escape with the cytokines and chemokines produced in the gut, travel along the mesenteric lymphatic system to the cisterna chyli, and subsequently enter the circulatory system. Thereby they have access to pulmonary circulation, which may lead to the local activation of dendritic cells and macrophages as well as T cell priming and differentiation. Another way to influence the pulmonary region might be through the migration of immune cells themselves, after priming and activation at the 1st site of antigen encounter, i.e. the gut mucosa. Although this theory explains the unilateral interaction, it is reasonable to speculate that this axis works the same way when it originates in the lung mucosa and lung lymph nodes.^[4] Moreover, nutrition can also affect both immune response and composition of our respiratory tract microbiota.^[5] In mice, high-fibre diet increased protection against allergic inflammation in the lung (reduced inflammatory cell infiltration), followed by a change in the gut and, to a lesser extent, the airway microbiota.^[6] The study also reported an increase in blood levels of circulating short-chain fatty acids (SCFAs), one of the major products of bacterial fermentation responsible of intestinal barrier integrity and known for its anti-inflammatory properties. However, no traces were found in the lung itself. On the contrary to allergic inflammation, a lack of an appropriate stimulus during the developmental phase of an immune response, as during infection, will disable a quick and effective immune reaction. This could result in undesirable consequences such as pathogen colonisation, increased susceptibility to infection, tissue damage, possible development of cancer and increased mortality.^[7,8] Therefore, it is clear that there is a complex network of distinct and precise stimuli that are required for executing a correct immune response. According to the gut-lung axis theory, these stimuli can originate in the gut, explaining the observed protective effect in the lung. Taking a huge step forward, the study of Routy et al (2018)^[9] evaluated the role of gut microbiota in responsiveness to anticancer treatment by immune checkpoint inhibitors (ICI) (PD-1/PD-L1). They showed that non-small cell lung cancer (NSCLC) patients that received antibiotic treatment (ATB) during 2 months before therapy had significantly decreased overall and progression-free survival. Similarly, ATB treatment was a predictor of ICI resistance, independent from other

prognostic markers. When faecal microbiota transfers using the stool from NSCLC patients responding or not responding to therapy were performed, inoculated germ-free (GF) mice showed the same phenomenon during ICI therapy against MCA-205 tumours. Mice receiving ICI therapy that were inoculated with a responder's stool showed delayed tumour growth and accumulation of antitumour lymphocytes in the tumour microenvironment. The stool of NSCLC patients responding to ICI therapy was found to be enriched in phylum *Firmicutes*, as well as distinct genera such as *Akkermansia*, *Ruminococcus*, *Alistipes*, etc. In further experiments with GF mice, *Akkermansia muciniphila* proved to be sufficient to restore ICI therapy responsiveness, both when inoculated alone or with the stool from non-responding NSCLC patients, rectifying the response. Likewise, it was the only species that induced reactivity from patient-derived Th1 and Tc1 in vitro, and that correlated with progression-free survival. Looking at these results, it is evident that the gut microbiota plays a crucial role in the host's homeostasis and that its fine-tuned composition counts for much more than was previously thought. However, data on this topic remain scarce but directed to a promising field of a new anti-lung cancer approach that the world population is yearning for.^[10] Unlike the local and systemic influence of the gut microbiota, the influence on and of the lung microbiota and its products has yet to be properly assessed, both in health and disease.^[11]

Therefore, to help to elucidate this new and extremely interesting field, we have decided to conduct a case-control observational study in patients with NSCLC. The study will include 2 groups of patients: 1st group Pchir, with patients eligible for treatment by surgery, and 2nd group Pct-chir, with patients eligible for a combined treatment consisting of neoadjuvant platinum-based chemotherapy followed by surgery.

The objectives of this study are:

- (i) to characterise the gut, lung and upper airway microbiota in these patients;
- (ii) to evaluate the homogeneity/heterogeneity between different microbiota within the same subject/group of patients;
- (iii) to evaluate the impact of the microbiota composition on immune and inflammatory status of the patient (evaluated in the gut, blood, lung);
- (iv) to evaluate the effect of chemotherapy on the site-specific microbiota (UAs, lung, gut).

While group Pchir will have only 1 time point for sample collection, group Pct-chir will have multiple time points. The latter will enable follow-up on changes in microbiota and immune markers relative to the treatment progression.

2. Methods and analyses

2.1. Ethics approval and dissemination

This protocol has been approved by the Committee for the Protection of Persons (CPP) Sud-Est VI, Clermont-Ferrand, France, and The French National Agency for Medicines and Health Products Safety (ANSM) (study ref. 2016-A01640-51). Because of the invasiveness of the sampling techniques, the requested control group was not approved by the CPP. The study was accompanied by amendment approved by ANSM in June 2018. The current protocol is entitled “Protocol MICA V3”, and presents an up-to-date version and the version in use. This study is registered with the Clinical Trials under ID: NCT03068663. The study's official name is: Characterisation of the microbiota

(gut, lung, and upper airways) in patients with non-small cell lung carcinoma: exploratory study (acronym: MICA). Written informed consent is obtained from all patients before enrolment in the study. The results are planned for presentation at conferences and publication in peer-reviewed journals in early 2019. All samples will be preserved for 15 years according to the practice of the sponsoring institution (Centre Jean Perrin). Samples will be available to other investigators if they want to perform complementary studies that consider NSCLC after additional consent obtained from patient. However, because of French regulations regarding patient information files, patients' data will not be available.

2.2. Study outcomes

As its primary outcome, this study will characterise the lung and UAs microbiota in 2 groups of 20 patients with NSCLC. Group Pchir will include patients eligible for surgery without chemotherapy. Group Pct-chir will include patients eligible for surgery after platinum-based chemotherapy. The UAs microbiota will be evaluated from saliva, while lung microbiota will be evaluated from 3 lung explants: "healthy" lung tissue, tumour and peritumoural tissue; and broncho-alveolar lavage fluid (BALF) from the tumour's proximity (same lobe). Following bacterial DNA extraction, microbiota will be analysed by qPCR and 16S ribosomal rRNA gene sequencing using the Illumina MiSeq platform.

Secondary outcomes of this study are set as follows:

- i) to study the effect of neoadjuvant chemotherapy on microbiota by evaluating:
 - a. the variation of the proportion of the phylum *Firmicutes* (as the phylum is highly represented in all of the different types of samples considered in this study),^[4]
 - b. the variation of the proportion of the bacterial genera per phylum in different types of samples (faeces, saliva, BALF, lung tissue/peritumoural tissue/tumour),
 - c. the concordance of genera between sample locations (e.g. saliva vs. BALF, healthy lung tissue vs. peritumoural tissue vs. tumour);
 - by qPCR and 16S rRNA gene sequencing;
- ii) to study the homogeneity/heterogeneity between lung, upper airways and gut microbiota for each and between both groups (evaluated by 16S rRNA gene sequencing and qPCR) and between different time points (group Pct-chir),
- iii) to evaluate immune/inflammatory status:
 - a. in the gut: by dosing β -defensin 2, human neutrophil peptides HNP1-3, calprotectin (ELISA) and SCFAs (gas liquid chromatography)
 - b. in plasma: by dosing plasmatic cytokines (Luminex), C-reactive protein (CRP) (ELISA) and immune cell phenotyping (flow cytometry)
 - c. in the lung: by immune cell phenotyping (flow cytometry) and characterisation of immune infiltrate in lung tumour biopsies obtained during the operation (by immunohistochemistry).

2.3. Patient recruitment

The study pre-considers all patients diagnosed with NSCLC and presented before the Thoracic Oncologic Committee of the Jean Perrin Centre, Clermont-Ferrand, France. Inclusion criteria are presented in Table 1. Inclusion in the study is consecutive and parallel for both groups. A written informed consent is obtained from each patient participating in the study before inclusion. Depending on their diagnosis, patients are included in one of the 2 groups: Pchir (patients eligible for surgery only), or Pct-chir (patients eligible for surgery after neoadjuvant platinum-based chemotherapy).

2.4. Patient and public involvement

Neither patients nor the Patient Committee were involved in the design of this study. If desired, patients can be informed of the study's results by the investigator or physician.

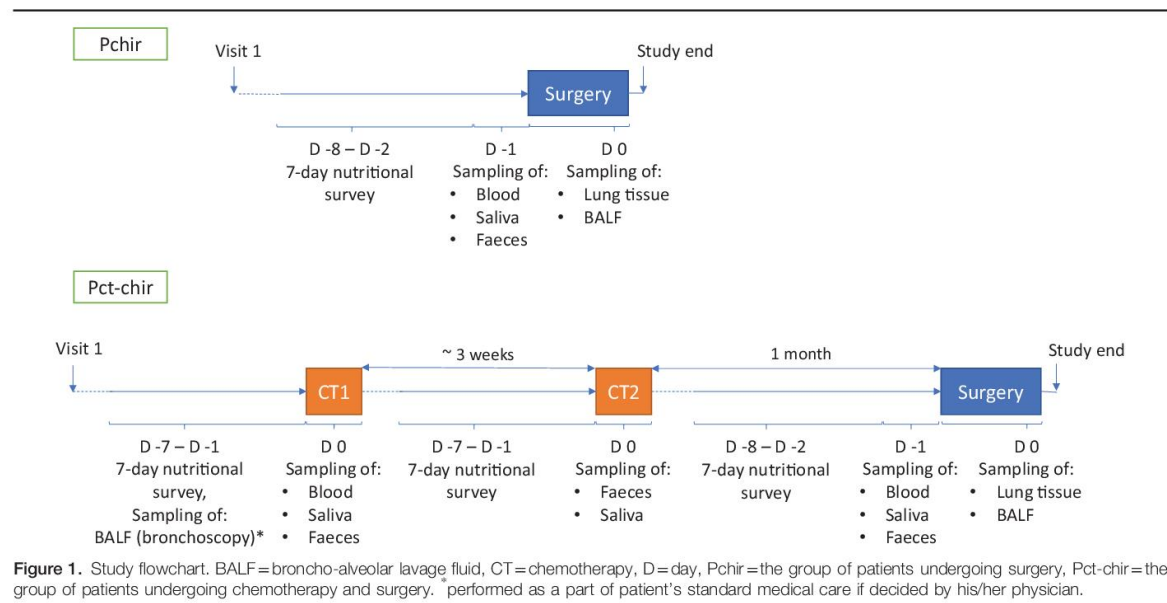
2.5. Trial design and timeline

This study is a case-control observational trial. Recruitment into the study started in May 2017 and will end in May 2019. The estimated complete duration of the study is 29 months, with an average follow-up period per patient of 1.5 and 3.5–4.5 months for the Pchir and Pct-chir groups, respectively. Intermediary analyses will take place after obtaining all samples from half of the patient quota (n = 20) regardless of the group, without interruption of the further recruitment to the study.

The trial design is presented in Figure 1. Eligible patients meet official study personnel at an outpatient appointment (Visit 1) where all the details of the protocol are thoroughly explained. At this visit, patients give their written consent to participate in the

Table 1
Inclusion and exclusion criteria for patients.

Inclusion criteria	Exclusion criteria
<ul style="list-style-type: none"> • NSCLC patient with an indication of surgery or neoadjuvant chemotherapy plus surgery • > 18 and < 80 years of age • BMI < 29.9 • Not-treated with antibiotics, corticoids or immunosuppressive drugs for at least the past 2 months • Signed written consent before enrolment in the study • Affiliated with the Social Security System 	<ul style="list-style-type: none"> • Cognitive difficulties • Refusal or inability to give clear consent to participate • Acute digestive or pulmonary infections in the past 2 months (requiring antibiotic treatment) • Inflammatory intestinal pathologies • Colostomy • Partial or complete gastrectomy • Previous oesophageal surgery • Previous otorhinolaryngeal cancer treated by radiotherapy or surgery • Inability to conform to the study's requirements • Deprivation of a right to decide by an administrative or juridical entity • Ongoing participation or participation in another study < 1 month ago • Ground-glass opacity



study. Depending on their clinical diagnosis, they are assigned to one of the 2 groups (Pchir or Pct-chir). Each patient is given a protocol summary, sampling instructions and corresponding number (depending on inclusion group) of tubes for saliva, boxes for faecal samples with anaerobic atmosphere generation bags and templates for a 7-day nutritional survey. Other information is also recorded, such as smoking status and history, weight or diet modifications in the last several months/years, cohabitation or interaction with animals in their childhood/present, the environment in which patients grew up/spent their life (country-side/city), exposure to certain pollutants, e.g. related to their profession, etc. This information will serve for better understanding of the individual's inflammatory status and microbial characteristics.

As presented in Figure 1, group Pchir has sampling concentrated around 1 event—surgery. At hospital admission (D-1 before surgery), samples of saliva, blood and faeces are collected together with the nutritional surveys patients made during the preceding week (D-7 to D-1). The BALF and lung tissue samples are collected during the surgery (D0) from the excised lobe.

Group Pct-chir (Fig. 1) has sampling concentrated around 3 events:

- 1st chemotherapy cycle (CT1)—the day of CT1, samples of saliva, blood and faeces are collected together with nutritional surveys patients made during the preceding week (same as D-1 for Pchir group). After sample retrieval, patients continue with their medical care procedure. Optionally, in the days preceding the CT1 as the part of their standard medical care, patients can undergo bronchoscopy. In this case, a part of retrieved liquid (BALF) is taken for microbiota characterisation and immune cell profiling.
- 2nd chemotherapy cycle (CT2)—CT2 is usually 3 weeks after CT1. The day of CT2, only saliva, faeces, and nutritional surveys are retrieved. After sample retrieval, patients continue with their medical treatment.

- Surgery—The time elapsed between CT2 and surgery is about 1 month, depending on the patient's overall health status. The sampling for surgery is exactly the same as for group Pchir. At hospital admission (D-1 before surgery), samples of saliva, blood and faeces are collected together with nutritional surveys patients made during the preceding week (D-7 to D-1). The BALF and lung tissue samples are collected during the surgery (D0) from the excised lobe.

The strong point of this trial is that study participation does not modify the patient's standard care treatment in any way. Invasive intervention, such as sampling of the lung tissue, is done during operation on the lung already removed from the patient. The BALF sampling during operation is performed directly on the dissected lobe, posing no additional inconvenience for the patient and drastically minimising UAs contamination, which is an important advantage to this study's concept. Likewise, sampling of the BALF at CT1 for group Pct-chir is done in the scope of a standard care procedure by bronchoscopy and it is not imposed by the study. However, the latter is also an inconvenience because there will be patients from group Pct-chir who will not undergo bronchoscopy with BALF sampling since it is not prescribed by his/her physician. Another thing to consider is also the difference in sampling technique of BALF. Sampling of BALF at CT1 for group Pct-chir is done by bronchoscopy, while sampling during surgery is done directly on the excised lobe for both groups. Therefore, the 1st BALF has a higher risk of contamination by UAs, while the 2nd BALF (during operation) should have no UAs contamination and better represent luminal microbiota specific of the tumour lobe. This will be taken into account during data interpretation, and if necessary, each time point will be characterised for itself.

In conclusion, in the group Pct-chir, the effect of chemotherapy on the microbiota of saliva and faeces and immune parameters of blood and faeces will be systematically assessed. Chemotherapy's effect on immune parameters and microbiota in BALF will be assessed only if obtained data in quantity and quality will permit

it (as explained above). When talking of lung tissue, “healthy” lung tissue, taken at distance from the tumour, will be considered as a control^[12] tissue for comparison with peritumoural and tumoural tissue, as well as with BALF. Also, characterisation will be done respective to the tumour type where possible (sufficient patient number with the same tumour type).

A demanded control group for the study was not authorised by the Ethics Committee, due to the invasiveness or evident inability to realise certain sampling steps (bronchoscopy, lung tissue sampling). Therefore, the focus will be on characterisation of the site-related microbiota and its connection to local and systemic immunity in NSCLC respective of the treatment group (Pchir or Pct-chir). Likewise, the question is whether there is an initial difference between group Pchir and group Pct-chir (before CT1), and what its nature is. Equally, how does chemotherapy modify group Pct-chir, and whether it becomes more alike or different from group Pchir regarding its different properties (microbial taxa ratios, inflammatory properties) when followed in time (from CT1 to surgery). Furthermore, obtained results considering microbiota composition and abundance, where possible, will address similar studies^[13–16] only in a descriptive matter and the same will be done with immune parameters.^[17–21]

3. Sampling and data recording

3.1. Nutritional survey

The dietary habits are evaluated for each patient for the 7 days preceding chemotherapy (Pct-chir) and/or surgery (Pchir and Pct-chir). At Visit 1 (see Fig. 1), all participants receive a detailed verbal explanation, written instructions and the survey with an example. They are asked to maintain their usual dietary habits during the survey period and to record as accurately as possible the amount, type and preparation of food and fluid consumed. If they consume commercial and ready meals, they are also asked to note brand names. The quantity of food or drink can be expressed in either precise measures (weight) or in common household measures, such as cups, tablespoons, etc. In the case of any questions or ambiguities, patients are encouraged to contact the study personnel. These data will help to estimate each patient's overall nutritional status and help to explain the microbiological analysis of faecal samples following the survey, as well as the patient's immune and inflammatory status. We anticipated that the patients might change their dietary habits during the chemotherapy duration, which is why recording was requested before each chemotherapy treatment and surgery, and during 1 week. The primary objective is to use what was recorded as complementary data to the faecal microbiota analysis (sampled the day after the end of each survey), to better explain the longitudinal modification of microbiota, if any. This is due to the fact that faecal microbiota can overcome significant changes in only a few days relative to a diet change.^[22]

3.2. Saliva

At Visit 1, after recording any evidence of oral health problems or injuries, each patient receives a tube for saliva collection (Sarstedt). It is necessary to fill the tube with a minimum of 1 mL of saliva (designated on the tube) on an empty stomach by the passive drooling method on the morning of hospital admission for the chemotherapy session (Pct-chir) and/or surgery (Pchir, Pct-chir) (Fig. 1). The sample is stored at -80°C for later bacterial DNA extraction and metagenomic sequencing.

3.3. Blood

Blood sampling is done following the patient's admission to hospital, in the day/hours preceding the prescribed clinical treatment, depending on the group (Fig. 1). A 10 mL of fresh blood are collected in EDTA treated tubes, where 500 μL of whole blood is immediately used for immune cell phenotyping by flow cytometry. The remainder is centrifuged for 10 min $2000 \times g$ at 4°C to obtain plasma, which is then aliquoted and stored at -80°C for further analyses (cytokine/interleukin analysis by Luminex, CRP dosage by ELISA).

3.4. Faeces

At Visit 1, each patient receives a sampling box, an anaerobic atmosphere generation bag (GENbag anaer, Biomérieux) and detailed printed instructions on how to handle the samples at his/her home. Faecal samples are collected following the 1-week nutritional survey (i.e. on the day of chemotherapy before the drug infusion or the day preceding surgery). In brief, sampling is done directly into the sampling box, and after removing the protective foil and placing the anaerobic atmosphere generation bag in the box, the box is closed firmly and placed in the cold ($+4^{\circ}\text{C}$). Patients are asked if they have the ability to transport the sample in an insulated bag to preserve cold conditions. If there is no such possibility, the study personnel supplies the patient with the requested bag. The sampling should be done within 12 hours preceding the hospital admission and sample retrieval. Therefore, patients are asked to do the sampling the morning of hospital admission if possible. If there are any problems, the patient is asked to contact the protocol personnel to ensure that the sample is processed in time. On reception, the sample is aliquoted $3 \times 1\text{ g}$ for bacterial DNA extraction, and $2 \times 5\text{--}10\text{ g}$ (depending on availability) for dosage of faecal calprotectin, β -defensin 2, HNP1-3 (ELISA) and SCFAs (gas liquid chromatography). Aliquots are stored immediately at -80°C until analysis. Approximately 1 g of fresh sample is used for bacterial culture of the main functionary groups of microorganisms (total anaerobic bacteria, mucin-degrading bacteria, lactic acid producing bacteria, sulphate-reducing bacteria and *Enterobacteriaceae*).

3.5. Lung tissue and BALF

Only for group Pct-chir, BALF is sampled at inclusion to the study. This sample is taken as a part of patient's standard care protocol if decided by his/her physician, and is not taken as an additional sample for this study. The BAL is performed by routine bronchoscopy procedure.

Sampling of lung tissue and BALF during surgery is performed for both groups, after partial or complete pneumonectomy. The removed lung tissue is placed in a sterile vessel and the tumour position is determined by palpation. A piece of healthy lung distal to the tumour, with a minimum size of $1\text{ cm} \times 1\text{ cm} \times 1\text{ cm}$, is then clamped. The clamp is left in place during the following procedure. The stich on the bronchus is cut away and using a sterile syringe the lung is inflated through the bronchus. Lavage is performed by instilling $2 \times 40\text{ mL}$ of sterile physiological saline. After each instillation, the maximum amount of liquid inside the bronchus is retrieved (8–10 mL in total), poured into a sterile 50 mL tube and placed immediately on ice, designated as “BALF”. At the end, the clamped wedge is cut off and designated as “healthy lung”. A slice of the tumour, with a minimal weight of 400 mg, containing the tumour cross-section is excised along with

peritumoural tissue, after which the 2 are separated based on histological difference. All tissues are frozen 1st in liquid nitrogen and then placed at -80°C for long-term storage until DNA extraction. The mirror piece of the excised tumour slice is stored in paraffin and later analysed by immunohistochemistry for characterisation of tumour infiltrate. A 3 mL of BALF are immediately used for immune cell analysis by flow cytometry and the remainder is stored at -80°C for later DNA extraction.

4. Methods and analyses

Saliva, “healthy” lung, peritumoural, and tumour tissue, BALF and faecal samples will be used for bacterial DNA extraction, followed by qPCR and 16S rRNA gene sequence analysis to establish microbial profiles. Fresh faeces samples will be used for bacterial culture, and frozen aliquots for dosage of faecal calprotectin, β -defensin 2, HNP1-3 (ELISA), and SCFAs (gas liquid chromatography). The BALF and plasma samples will both be analysed by flow cytometry (immune cell phenotyping), while plasma will also be used for cytokines (Luminex) and CRP (ELISA) dosage. Tumour tissue stored in paraffin will be used for the analysis of immune infiltrate by immunohistochemistry. Each procedure is explained in detail in the following sections.

4.1. Nutritional status

Nutritional data for each patient will be analysed using the Nutrilog 2.3 software package, a computerised database (Proform) that calculates food composition from the French standard reference.^[23]

4.2. Cytokines

Stored plasma will be analysed for cytokines corresponding (but not restricted) to the following profiles: Th1, Th2, Th17, Treg. Samples will be analysed using Luminex kits: HSTCMAG-28SK, HTH17MAG-14K and TGFBMAG-64K-01 (Merck Millipore). Analyses will be conducted by the phenotyping service of CREFRE, Toulouse, France.

4.3. Evaluation of tumour immune infiltrate

Immunohistochemistry will be performed on tumour tissue using the specific antibodies to detect subpopulations of immune cells as follows: cytotoxic T-lymphocytes (anti-CD8, clone SP16, ThermoFisher Scientific), regulatory T-lymphocytes (anti-FoxP3, clone SP97, ThermoFisher Scientific), B-lymphocytes (anti-CD20, clone SP32, Cell Marque). The immune response checkpoint axis PD-1–PD-L1 will be assessed by anti-PD-1 (clone NAT105, Cell Marque) and anti-PD-L1 (clone 28-8, Abcam). All staining will be performed by a fully automated, standardised procedure (Benchmark XT, Ventana/Roche). The number of lymphoid cells expressing each antigen, except PD-L1, will be determined within 5 consecutive $\times 40$ microscopic fields, starting from the invasive front toward the tumour centre, used as a parameter reflecting the tumour’s quantity of a given immune cell subpopulation. PD-L1 will be assessed for both immune and tumour cells and reported as the percentage of each population expressing the antigen.

4.4. Immune cell phenotyping

Immune cell phenotyping will be performed on fresh samples of blood (0.5 mL) and BALF (3 mL) by flow cytometry. Leukocytes will be obtained after haemolysis (solution of 155 mM NH_4Cl , 12

mM NaHCO_3 , 0.1 mM EDTA) for 15 min at room temperature, followed by 10 min centrifugation at $600 \times g$. Before centrifugation, BALF will be filtered through a porous gauze to eliminate mucus and reduce the viscosity. Lymphocyte subpopulations will be phenotyped using the following antibodies: anti-CD3-VioBlue, anti-CD4-APC-Vio770, anti-CD25-APC, anti-CD127-VioBright FITC, anti-CD183 (CXCR3)-PE-Vio770, anti-CD294 (CRTH2)-PE, anti-CD196 (CCR6)-PE-Vio615, anti-CD15-FITC, anti-CD62L-PE, anti-CD11b-PE-Vio770 and Viability 405/520 fixable dye, all purchased from Miltenyi Biotec. A T lymphocytes CD4^+ will be characterised as $\text{CD3}^+\text{CD4}^+$ cells. Subpopulations of T lymphocytes CD4^+ will be characterised as follows: Th1 as $\text{CD3}^+\text{CD4}^+\text{CD183}^+$, Th2 as $\text{CD3}^+\text{CD4}^+\text{CD294}^+$, Th17 as $\text{CD3}^+\text{CD4}^+\text{CD196}^+$, Treg as $\text{CD3}^+\text{CD25}^+\text{CD127}^-$ and neutrophils as $\text{CD15}^+\text{CD11b}^+\text{CD62L}^+/\text{CD15}^+\text{CD11b}^-\text{CD62L}^+$ for “tethering” form, and $\text{CD15}^+\text{CD11b}^+\text{CD62L}^-$ for active form. Due to high debris background in BALF samples, utilisation of the Viability dye is essential and utilisation of intracellular dyes is excluded. The data will be acquired using LSRII, BD Biosciences.

4.5. Inflammatory/antimicrobial markers and short chain fatty acids (SCFAs) analysis

Faecal samples will be analysed for calprotectin (kit Calprest NG, Eurospital, with an adaptation on BEP2000 (Siemens)), β -defensin 2 (β Defensin 2 ELISA Kit, Immundiagnostik, Bensheim), and HNP1-3 (human HNP1-3 ELISA Kit, Hycult biotech). All 3 markers will be measured in the Laboratory of Functional Coprologie, GH Pitié-Salpêtrière, Paris. The C-reactive protein will be measured in plasma by CRP human ELISA kit (Enzo Life Sciences). The SCFA concentration will be dosed after water extraction of acidified faecal samples using gas liquid chromatography (Nelson 1020, Perkin-Elmer) in the Commensals and Probiotics-Host Interactions Laboratory, Micalis Institute, INRA UMR 1319, France.

4.6. DNA extraction

A DNA extraction on all samples will be performed in batches to reduce the possibility of manipulation errors between extractions.

4.6.1. Sample pre-treatment. Lung tissue. Lung tissue will be taken directly from liquid nitrogen, broken into smaller pieces with a mortar and pestle, and homogenised in Hank’s balanced salt solution (HBSS) (Sigma-Aldrich) in gentleMACS M tubes (Miltenyi Biotec). The ratio of buffer volume:sample weight will be determined for each sample, and adapted volume will be used for each of the following steps. The programs used will be those adapted for lung and tumour tissue (Miltenyi Biotec). The homogenate obtained will be treated with collagenase D (Sigma-Aldrich) (2 mg/mL final concentration) at 37°C for 15 min, followed by 10 min at $2000 \times g$ at room temperature (RT). The pellet will be resuspended in 2–5 mL (depending on the initial sample weight) of mammalian cell lysis buffer (MCLB),^[24] and repeatedly vortexed for 5 min at RT. The reaction will be stopped with adding an equal volume of neutralisation buffer.^[24] After 2 washes with PBS (Sigma) ($2000 \times g$ for 10 min), the pellet will be used for DNA extraction using the adapted protocol of Godon et al.^[25]

Saliva and BALF. Saliva and BALF will first be brought to RT and vortexed. 1 mL of saliva and 5 mL of BALF will be used for DNA extraction. Whole BALF will be used for extraction, to minimise the loss of bacterial communities.^[26] BALF will be centrifuged ($7000 \times g$, 10 min) and 3 mL of MCLB will be added to the pellet, while saliva will be treated directly with 1 mL of MCLB. Both will be vortexed for 5 min at RT, followed by

addition of neutralisation buffer. DNA extraction will be performed directly on the pellet after centrifugation at $7000 \times g$ for 10 min at RT and a washing step (PBS).

Faeces. Faecal samples will have no pre-treatment and extraction will begin directly on frozen samples.

4.6.2. DNA extraction. DNA extraction will be performed by using the adapted protocol of Godon et al.,^[25] i.e. International Human Microbiome Standards Standard Operating Protocol for Fecal Samples (IHMS SOP) 07 V1. Briefly, 4M guanidine thiocyanate and 10% N-lauroyl sarcosine will be added directly on frozen samples or pellets for 10 min at RT. After the addition of 5% N-lauroyl sarcosine and homogenisation by vortexing, the samples will be incubated for 1 h at 70°C. All of the samples will be transferred to Lysing Matrix B tubes (MPBio) and homogenised using FastPrep-24 Instrument (MPBio), 4×45 s at 6.5 ms^{-1} . Between each cycle, the samples will be cooled on ice for 2 min. One micro-spoon of polyvinylpyrrolidone will be added to each tube, followed by vortexing and centrifugation for 3 min at $18,000 \times g$. The supernatant will be removed and placed in a new 2 mL tube and the pellet will be washed with TENP and centrifuged for 3 min at $18,000 \times g$, and the new supernatant will be added to that which was harvested previously. The pooled tube will be centrifuged for 1 min at $18,000 \times g$ and the supernatant will be transferred to a new 2 mL tube. One volume of isopropanol will be added to the supernatant, gently mixed by turning the tube and incubated for 10 min at RT. After centrifugation for 5 min at $18,000 \times g$, the pellet will be resuspended in 0.1 M phosphate buffer, pH 8, and 5 M potassium acetate and incubated overnight at 4°C. The samples will then be centrifuged for 30 min at $18,000 \times g$ and 4°C. The supernatant will be transferred to a new 2 mL tube, and after the addition of RNase (final concentration 40 $\mu\text{g}/\text{mL}$), incubated at 37°C for 30 min. Nucleic acids will be precipitated with absolute ethanol and 3 M sodium acetate, followed by centrifugation at maximum speed for 3 min. The pellet will be washed with 70% ethanol, dried and resuspended in 100 μL of TE buffer. DNA quality and concentration will be estimated by agarose gel electrophoresis and nanodrop (NanoDrop ND-1000) measurement, respectively.

Considering the low biomass samples, background controls have been made throughout the sampling and extraction process. The physiological serum used to perform BAL is 1st sampled with the same syringe that is afterward used for lavage from the same vessel containing physiological serum. This sample is used as a “negative sampling control”. During the DNA extraction, milliQ water is used as a “negative background control” sample, treated will all the reagents and passing all the procedures along with the real samples. These “negative” samples will be analysed along with the real samples.

All the reagents used in DNA extraction and sample pre-treatments were either autoclaved, filtered through 20 μm filters or purchased sterile. All the tools and pipettes were thoroughly washed and disinfected between extractions of different sample types, to minimise the transfer from high biomass samples. Also, DNA extraction from lung tissue samples (3 samples per patient) was randomised (each extraction “batch” never contained only one sample type from different patients or all the samples from the same patient), to minimise the “batch” effect.

4.7. Molecular analyses of microbiota

4.7.1. The 16S rRNA gene sequencing. The genomic DNA from saliva, faeces, BALF, “healthy” lung, peritumoural and tumoural tissue, and negative controls will be analysed by

sequencing of the bacterial 16S rRNA gene by DNAVision, Belgium, using Illumina MiSeq technology. After PCR amplification of the targeted region V3–V4, libraries will be indexed using the NEXTERA XT Index kit V2. The sequencing is carried out in paired-end sequencing (2×250 bp) by targeting an average of 10,000 reads per sample. Software used for bioinformatic analysis will be QIIME (Quantitative Insights Into Microbial Ecology), with a cut-off value of 5000 reads per sample for analysis. For each sample the following will be determined:

- alpha and beta diversity,
- comparison of alpha diversities based on a 2-sample *t* test using non-parametric (Monte Carlo) method,
- statistical significance of sample groupings using distance matrices (Adonis method),
- comparison of OTU frequencies across sample groups (comparison is performed at OTU, Phylum, Class, Order, Family and Genus level)

Multiple comparisons will be realised, both between different grouping criteria and between samples.

Considering the low taxonomic levels (Species), sequencing is best used as an indicative tool for further analyses by qPCR.

4.7.2. qPCR. In our study, qPCR will be done in 2 phases:

- Pre-16S sequencing analysis

In this phase, qPCR will have 2 purposes: 1st, to confirm and further characterise the bacterial functionary groups in faeces evaluated by bacterial culture (providing the information of viable bacteria inside specific functionary group); and 2nd, to quantify pathogens/commensals in respiratory and intestinal system, known to be implicated in tumourigenesis/pro or anti-inflammatory reactions^[27–32] using specific primers.

The qPCR will provide information of absolute quantity of taxa/species of interest in each sample, which is information that cannot be obtained by sequencing (only relative abundance).

- Post-16S analysis

In our study, the sequencing has the purpose of sample “screening”. It will give us an idea of the composition of the microbial communities from different sites and originating from different conditions (patient with tumours eligible for chemotherapy/surgery) based on minimum of 5000 reads. However, sequencing stays a technique to determine relative abundance. Therefore, once the overall composition of each sample is determined, we will proceed with:

- quantification of the specific outer taxonomic units (OTUs)/ taxa we determine as relevant in either relative or normalised abundance not analysed during pre-16S analyses
- enlarging the primer list specific for the new discovered OTUs of interest

The current list of primers optimised for our study is shown in Table 2. Each primer couple is tested for specificity on 60 referent species. The QPCR will be done using the Rotor-Gene Q machine (Qiagen). Additional primer couples will be tested and optimised if found necessary, as explained.

4.8. Statistical analysis plan

4.8.1. General information. This study is exploratory and main outcomes address the description of microbiota characteristics in lung cancer patients. Three microbiota are concerned (gut, lung, and saliva) and data are collected at different times in patients treated by neoadjuvant chemotherapy (group Pct-chir). For other patients (group Pchir), different microbiota are sampled at only 1 time point, at surgery.

Table 2
The current list of qPCR primer couples.

Target group		Primer sequence (5' – 3')	Reference
Total bacteria	F	CGGTGAATACGTTCCCGG	Furet et al, 2009 [33]
	R	TACGGTACCTTGTACGACTT	
<i>Bacteroidetes</i>	F	AACGCTAGCTACAGGCTTAACA	Dick & Field, 2004 [34]
	R	ACGCTACTGGCTGGTTCA	
<i>Actinobacteria</i>	F	TACGGCCGCAAGGCTA	Trompette et al, 2014 [6]
	R	TCRTCCCCACCTTCCTCCG	
<i>Firmicutes</i>	F	GGAGYATGTGGTTAATTCAAGCA	Guo et al, 2008 [35]
	R	AGCTGACGACAACCATGCAC	
<i>Gammaproteobacteria</i>	F	CMATGCCGCGTGTGTGAA	Mühling et al, 2008 [36]
	R	ACTCCCAGGCGGTCDACTTA	
<i>Bacteroides/Prevotella (Bacteroidales)</i>	F	CCTWCGATGGATAGGGGTT	Layton et al, 2006 [37]
	R	CACGCTACTGGCTGGTTCCAG	
<i>Lactobacillus/Leuconostoc/Pediococcus</i>	F	CGCCACTGGTTCYTCATATA	Furet et al, 2009 [33]
	R	AGCAGTAGGGAATCTCCA	
<i>Blautia</i> genus	F	GTGAAGGAAGAAGTATCTCGG	Kurakawa et al, 2015 [38]
	R	TTGGTAAGGTTCTTCGCGTT	
<i>Veillonella</i> genus	F	GRAGAGCGATGGAAGCTT	Tana et al, 2010 [39]
	R	CCGTGGCTTTCTATTCC	
<i>Neisseria</i> genus	F	CTGTTGGGCARCWTGAYTGC	Yan et al, 2015 [40]
	R	GATCGGTTTTRTGAGATTGG	
<i>Fusobacterium</i> genus	F	AAGCGCGCTAGGTGGTTATGT	Dalwai et al, 2007 [41]
	R	TGTAGTCCGCTTACCTCTCCAG	
<i>Bacteroides thetaiotaomicron</i>	F	GACCGCATGGTCTTGTATT	Haugland et al, 2010 [42]
	R	CGTAGGAGTTGGACCGTGT	
<i>Bifidobifida wadsworthia</i>	F	CGTGTGAATAATGCGAGGG	McOrist et al, 2001 [43]
	R	TCTCCGGTACTCAAGCGTG	
<i>Akkermansia muciniphila</i>	F	CAGCACGTGAAGTGGGGAC	Collado et al, 2007 [44]
	R	CCTTGGCGTTGGCTCAGAT	
<i>Escherichia coli</i>	F	CATGCCGCGTGTATGAAGAA	Huijsdens et al, 2002 [45]
	R	CGGGTAACGTCAATGAGCAAA	
<i>Faecalibacterium prausnitzii</i>	F	GGAGGAAGAAGGCTCTCGG	Ramirez-Farias et al, 2008 [46]
	R	AATTCGGCCTACCTCTGCACT	
<i>Blautia (Ruminococcus) gnavus</i>	F	GGACTGCATTTGGAAGTGTGAG	Le Leu et al, 2015 [47]
	R	AACGTCAGTACATCGTCCAGAAAG	
<i>Ruminococcus torques</i>	F	GCTTAGATTCTTCGGATGAAGAGGA	Le Leu et al, 2015 [47]
	R	AGTTTTTACCCCGCACCA	
<i>Bifidobacterium bifidum</i>	F	CCACATGATCGCATGTGATTG	Malinen et al, 2005 [48]
	R	CCGAAGGCTTGTCTCCAAA	
<i>Enterococcus hirae</i>	F	GGCATATTTATCCAGCACTAG	Dallière et al, 2016 [49]
	R	TAGCGTACGAAAAGGCATCC	
<i>Pseudomonas aeruginosa</i>	F	CCAGCCATGCCGCGTGTGTGA	Silva-Junior et al, 2016 [50]
	R	GTTGGTAACGTCAAAAACAGCAAGG	
<i>Streptococcus pneumoniae</i>	F	ACGCAATCTAGCAGATGAAGCA	Chien et al, 2013 [51]
	R	TCGTGCG TTTTAATTCAGCT	
<i>Haemophilus influenza</i>	F	AGCGGCTGTAGTTCCTCAACA	Fukumoto et al, 2015 [52]
	R	CAACAGAGTATCCGCCAAAAGTT	
<i>Fusobacterium nucleatum</i>	F	CAAGCGGTGGAGCATGTG	Fukumoto et al, 2015 [52]
	R	CTAAGATGTCAACGCTGGTAAGG	
<i>Moraxella catarrhalis</i>	F	GGTGAGTGCCGCTTTTACAAC	Fukumoto et al, 2015 [52]
	R	TGTATCGCCTGCCAAGACAA	
<i>Klebsiella pneumoniae</i>	F	CGGGCGTAGCGCGTAA	Fukumoto et al, 2015 [52]
	R	GATACCCGCTTACATTAACAG	

In patients treated by chemotherapy, analysis of variations of microbiota induced by chemotherapy will be possible by evaluation of changes in proportions over time. On the other hand, the analysis of microbiota with/without previous chemotherapy will only be descriptive, as the design is not compatible with a non-biased comparison of both groups.

4.8.2. Sample size. The sample size calculation is based on the study of Montassier et al, 2015,^[53] where the abundance of the principal phylum *Firmicutes* in faecal microbiota decreased by

approximately 30%, with an FDR-corrected *P*-value of .0002. Considering these results, 20 patients in the chemotherapy group should allow us to detect a similar variation in our samples, at least in the faecal microbiota. For group balance, the same sample size of 20 was retained for the surgery-only group.

4.8.3. Description of patients' characteristics. Patients' characteristics will be described using standard distribution parameters: counts, range, mean/median, confidence intervals, standard deviation/interquartile range for quantitative parameters and, for

categorical ones, counts and frequencies. This description will also be made by treatment group (Pchir/Pct-chir).

4.8.4. Description of microbiota. Microbiota characteristics consist in several hierarchical steps including:

- Phylum: 4 main phyla are found in both lung and intestinal microbiota (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*). *Firmicutes* are supposed to represent ~80% of the intestinal microbiota biomass^[54] and ~40% of the lung microbiota.^[55] The proportion of each component will be described by its proportion of the biomass in %.
- Main classes of bacteria per phylum: these classes gather bacteria that share important characteristics and functions (e.g. *Bacilli*, *Clostridia*, *Gammaproteobacteria*, etc.). Composition of the microbial communities from different sites and originating from different conditions will be quantified (alpha and beta diversity, relative proportions).
- Inside classes, description by order, family and genus level will be performed when contributory on a biological plan.

Heterogeneity between microbiota will be studied. The comparison of proportions of phyla, or by other taxonomic level will be performed to evaluate if specific adaptation characterises the 3 microbiota and their components. The ANOVA will be used to perform inter-patient comparisons. The FDR correction will be applied when analyses are conducted within phyla.

4.8.5. Comparison of microbiota before/after chemotherapy. This comparison will be performed for each site-specific microbiota. Proportions of main phyla will be compared using ANOVA (mixed model) to check if an independent chemo-effect can be objectivised, adjusting on patients and phyla (without FDR correction).

Comparisons of taxonomic levels below phylum before/after chemotherapy will be performed on relevant components with an FDR correction. These comparisons will be performed on both alpha and beta diversity. Univariate paired parametric or non-parametric tests (Student *t* test, Mann-Whitney *U* test, etc.) will be used here.

4.8.6. Comparison of microbiota between the 2 treatment groups. These comparisons will use the same tests as in the previous paragraph, except tests will not be paired.

4.8.7. Relationship between inflammatory status and microbiota. Several cytokines will be measured in blood samples. Each result will consist in a concentration of cytokine. The relationship between microbiota and inflammation will be tested using Pearson (or Spearman rank) correlation coefficient, using FDR correction.

4.8.8. Tumoural immune infiltration. Immune reaction will be described by percentage by lymphocyte type. These proportions will be compared to corresponding microbiota characteristics: for example lung microbiota and lung tumour. Statistical association between these parameters will be tested as in the previous paragraph.

4.8.9. Complementary analyses. Complementary analyses will be performed if particular biological issues can be better described.

All statistical analyses will be performed using R-software version 3.5.0 or later (R-Project, GNU GPL). Tests will be 2-sided and the significance threshold is set at 0.05, after FDR correction where needed (as for the analyses concerning taxonomic ranks below phylum). Data may be missing due to possible loss of

follow-up between inclusion and end of study. A description of the missing data and associated reasons will be given.

4.9. Data monitoring committee

A data monitoring committee is not needed in this study since this is an observational trial and there are no intervention or security risks for patients.

5. Discussion

5.1. What is known

Lung cancer is a leading cause of death by cancer worldwide, responsible for 1,761,007 or 23.1% deaths in 2018 according to the WHO.^[56] It is also the most frequent cancer in men and the 3rd most frequent in women.^[56] While well characterised regarding its aetiology, morphological, and molecular properties,^[57–59] much less is known regarding its relationship with lung microbiota, and almost nothing regarding its connection to distant sites such as the gut and gut microbiota. This lack of studies is self-explanatory when one knows that not so long ago lungs were considered sterile except in case of infection.^[55,60] Recently, however, there is an emerging idea of more “systemic” influence of the gut microbiota, and its connection to the immune system beyond the local effect.^[3,60–62] A few teams made a huge leap in elucidating the role of the gut microbiota in chemotherapy and anticancer treatment, including lung cancer.^[63–67]

5.2. What is new

Based on these studies and the questions unanswered, we designed a case-control observational trial underlining a multi-aspect approach to the patient. In each of our subjects, we decided to characterise the microbiota of different sites (UAs, gut, and lung microbiota), in parallel with immune profile characterisation (local and systemic) while taking into account the patient’s life style (nutrition, smoking status, profession, etc.). Examination of these factors in patients undergoing chemotherapy before surgery enables a direct follow up of these parameters correlated with the treatment phase (to our knowledge, this has never been reported for lung cancer before). Moreover, lung microbiota at surgery is sampled in 2 ways: by performing broncho-alveolar lavage (BAL) directly on the excised lung lobe (to eliminate possible UAs contamination and obtain the maximal microbial concentration for analysis), and by sampling lung tissue at 3 sites: “healthy” tissue distal to tumour (used as a control tissue),^[12] peritumoural tissue, and tumour itself. This enables sampling of both luminal and tissue/cell-bound bacteria which, according to known studies, do not share the same microbial composition.^[26] To our knowledge, at present there is no study of lung cancer that examines the microbiota of peritumoural tissue, and even less in 4 different lung sample types. Also, no study performed BAL directly on the tumour lobe without passing through the UAs.

5.3. Choice of analyses

As previously reported, certain bacterial species can modify our immune responses differently, such as *Faecalibacterium prausnitzii* or on the other hand *Fusobacterium nucleatum*, as well as the whole cluster (*Clostridia* cluster XIV).^[68,69] Therefore, tumour lymphocyte infiltration and lymphocyte composition in the broncho-alveolar lavage fluid (BALF) will be examined and

closely looked at for its relationship with sampled microbiota. Likewise, each tumour is characterised according to the TNM stage classification^[70] and histological properties. Tumour architecture, localisation in the lung, and disease severity are expected to dynamically interact with microbial composition *in situ* and immune profile, as seen in similar pathologic states of the lung (obstruction of the normal lung architecture, creation of anaerobic thermal pockets in the case of bronchial obstructiveness, immunogenicity of the tumour, promotion of neutrophil recruitment, inflammation).^[12,15,71–73] Difference of the microbiota composition between tumour samples of adenocarcinoma and squamous cell carcinoma has already been evidenced by Yu et al.^[15] Interestingly, salivary microbiota is also proven to correlate with NSCLC type.^[40] Therefore, similar analysis direction will be taken with our salivary and lung samples.

As mentioned, intestinal microbiota has both local and systemic influence on its host. According to the gut-lung axis theory,^[4] bacteria or their products might have systemic effects, and therefore, have an effect on the lung microbial composition and immune response. For this reason, faecal microbiota will be characterised, as well as faecal SCFAs concentrations (known products of bacterial fermentation and immune modulators/protectors of the intestinal barrier).^[74] The SCFAs might be potential mediators of the gut's "long-distance" influence by direct effect on the target site or indirectly via gut/circulating immune system stimulation. As intestinal microbiota is shown to adapt very quickly to the changes in nutrition, as well as its influence on SCFAs concentrations,^[22,75] nutritional records before each faecal sampling will be taken into account. We will not only determine the composition of faecal microbiota, but also its "quality" and influence on intestinal health by dosage of bacteriocins (HNP1-3, β -defensin 2), and calprotectin as inflammatory marker.^[76] Broad-spectrum cytokine profiling and immune cell phenotyping in the blood will be used to evaluate systemic immune status. This holds particular importance as connection to circulating IL-6 and IL-8 was previously reported in lung cancer,^[76,77] but also to intestinal SCFA concentrations.^[78]

As explained, group Pct-chir will enable follow-up on multi-site microbiota and immune status during different treatment phases (Fig. 1). Since the biggest problem of chemotherapy, despite its efficacy against tumour cells, is its non-selectivity (effecting epithelial layers and mucosae),^[79–81] we expect to see changes in all 3 types of microbiota—salivary, faecal, and lung, as all are closely related to epithelial and mucosal layers. Similarly, immune characteristics should be altered following the chemotherapy and above-mentioned changes in microbiota (but also vice versa—the affected immune system will change its interaction with microbiota, thus modifying it). Finally, we could hypothesise that different initial properties of the tumour (why the patient is prescribed chemotherapy or not in the 1st place) might divide 2 patient profiles (Pchir vs. Pct-chir 1st time point) regarding both multi-site microbial and immune/inflammatory characteristics.

5.4. Final word

To conclude, our results will be one of the 1st to give a better understanding of the close and intense interaction between the microbiota of different, yet communicating sites and their interaction with the immune system in patients suffering from lung cancer (the world's number 1 cause of death by cancer).^[82]

The strength of this study design is data collection through multiple non-invasive techniques that can be incorporated into the standard medical care and treatment of the patients. Another strong point is a multi-site approach towards each patient: lifestyle, nutrition, immune status, and microbial composition are assessed using different and complementary techniques (e.g. faecal microbiota will be assessed by techniques of molecular biology via qPCR and sequencing, but also by bacterial culture, and in the aspect of individual's nutrition). The main limitation is lack of the "healthy" control group, not authorised by Ethics Committee because of the invasiveness of the sampling techniques for healthy subjects. Therefore, previously published data on healthy subjects and similar cohorts will be addressed only in a descriptive matter, while we will focus more on relational aspects (e.g. interaction between site-specific immunity and its microbiota).

We hope that our results will help in setting the basis for developing more personalised or "alternative" approaches in lung cancer treatment, better characterisation of patient's status and diagnosis, as well as in finding ways of improving chemotherapy tolerance and effectiveness (complementary pre-biotics, probiotics or symbiotics).

Author contributions

The RB and EF wrote the manuscript. MF and EF proposed the study design and protocol preparation. JYB and ET were involved in the preparation of protocol amendments. MPV was involved in the study design. ABD and NRR led the study design and protocol preparation. FK wrote the statistical analysis plan. MF was responsible for patient recruitment, surgery procedure and lung sampling during surgery. Preparing the study design, sample and data collection, management, analyses and decision to submit the report for publication is the responsibility of RB, EF and MF. All authors have reviewed this manuscript.

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Annex 2

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*Review Article***Desired Turbulence? Gut-Lung Axis, Immunity, and Lung Cancer**

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The microbiota includes different microorganisms consisting of bacteria, fungi, viruses, and protozoa distributed over many human body surfaces including the skin, vagina, gut, and airways, with the highest density found in the intestine. The gut microbiota strongly influences our metabolic, endocrine, and immune systems, as well as both the peripheral and central nervous systems. Recently, a dialogue between the gut and lung microbiota has been discovered, suggesting that changes in one compartment could impact the other compartment, whether in relation to microbial composition or function. Further, this bidirectional axis is evidenced in an, either beneficial or malignant, altered immune response in one compartment following changes in the other compartment. Stimulation of the immune system arises from the microbial cells themselves, but also from their metabolites. It can be either direct or mediated by stimulated immune cells in one site impacting the other site. Additionally, this interaction may lead to immunological boost, assisting the innate immune system in its antitumour response. Thus, this review offers an insight into the composition of these sites, the gut and the lung, their role in shaping the immune system, and, finally, their role in the response to lung cancer.

1. Introduction

The microbiota is a consortium of different microorganisms that includes bacteria (microbiota), fungi (mycobiota), viruses, and protozoa [1] residing on the skin, and in the oral, pulmonary, urogenital, and gastrointestinal (GI) cavities—with the gastrointestinal having the highest density of microbiota. Weighing approximately 1.5 kg, microbial residents in the GI tract outnumber human cells 10-fold and genome size 100-fold. Their functional importance for the

host is undeniable involving functions that range from breaking down complex dietary polysaccharides [2] to competing with pathogens and modulating the mucosal and immune system in general [3]. Gut dysbiosis is now considered to be an underlying cause for a wide range of GI diseases and an emerging number of non-GI conditions such as obesity and cardiovascular disease, as well as a range of psychiatric diseases [4, 5]. Recent studies have also reported the effects that the intestinal microbiota exerts on the lungs. This has been referred to as the “gut-lung axis,” which in most cases

is mediated by inflammation involving the translocation of bacteria and bacterial products across the GI tract barrier and into blood vessels [6]. However, data on this topic is scarce. Studies of the lung microbiota and its interconnection with other systems and organisms are an emerging field, which is rapidly accumulating evidence to demonstrate that the lungs are not in fact sterile but contain distinct microbial communities [7]. Moreover, it appears that chronic lung diseases such as cancer are linked to a dysbiotic airway microbiota and commonly occur alongside GI disorders [8, 9]. Likewise, individuals with irritable bowel syndrome sometimes have impaired lung function [10]. This leads us to the conclusion that the axis between lung and gut can be considered bidirectional.

In this review, we give an overview of the composition of both the gut and the lung and describe the interaction between the immune system and microbiota using the intestinal tract as an example. In the case of the lungs, we are still only able to speculate about any similarity. We also examine immune stimulation of the gut to observe the effects on lung immunity, inflammation, and lung cancer, and finally, we discuss how these two sites might “cooperate” to achieve a productive immune and anticancer response.

2. Gut Microbiota

The evolution of an individual's microbiota begins at birth, with its composition becoming relatively stable after the age of two and remaining so throughout life. The GI tract is populated by more than 1,000 bacterial species. At the level of the phylum, the composition of the microbiota is similar in most healthy people. Over 90% of bacterial cells are Firmicutes and Bacteroidetes, followed by Actinobacteria, Proteobacteria, and Verrucomicrobia, together constituting 99% of the overall commensal microbiota [11]. Around 60 species have been identified as the “core” microbiota, mostly bacteria from the *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Ruminococcus*, and *Faecalibacterium* genera, as well as a few others [11]. A summary overview of the most prevalent human GI microbiota is shown in Table 1.

Strongly correlating to long-term diet [24], different enterotypes can also be identified through a variation in the levels of one of the three most abundant genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3), which is often less clearly distinguished [25].

2.1. The Impact of the GI Microbiota on Host Mucosal Immunity. The microbiota is now considered key to the proper development, maturation, and reactivity of the immune system [6, 26]. Microorganisms serve as an inexhaustible source of microorganism-associated molecular patterns (MAMPs) as well as pathogen-associated molecular patterns (PAMPs). The two are recognizable on the host's cells through pattern-recognition receptors (PRRs), which include toll-like receptors (TLRs) and nucleotide-binding receptors (NODs) [27]. TLRs are conserved receptors of the innate immune system that recognize MAMPs and PAMPs among other molecules, evoking different immunological reactions depending on the type of the cell, ligand, and the receptor itself (some of the

most common pairs are shown in Figure 1(a)). TLRs, which are in direct contact with the intestinal lumen, are found not only in intestinal epithelial cells (IECs) but also on immune cells within the lamina propria, such as macrophages, dendritic cells (DCs), B cells, T cells, and stromal cells. In IECs, TLR activation by microbial ligands results in epithelial cell proliferation and the expression of antimicrobial peptides, and secretion of immunoglobulin A (IgA) produced by plasma cells in lamina propria, into the gut lumen [23], as well as the expression of antimicrobial peptides. All of the above lead to enhanced intestinal barrier function and limit the possibility of microbial breach. Interestingly, some of the TLRs, such as TLR2 and TLR4, are inhibited by IECs intracellular Toll-interacting protein (TOLLIP) (Figure 1(b)) when the signal comes from the intestinal lumen, which suggests a selective inflammatory response reserved to microbes that have breached the intestinal barrier [23]. NOD-like receptors, or nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs), are cytoplasmic equivalents of TLRs that detect bacterial PAMPs entering the mammalian cell (Figure 1(b)). They are especially important in tissues where TLRs are expressed at low levels, for example, in GI tract epithelial cells where the cells are in constant contact with the microbiota, and the expression of TLRs must be downregulated to avoid overstimulation [28].

Commensal microorganisms can enter intestinal lamina propria in several ways: through an opening in the barrier as a result of injury or through active sampling by DCs or M cells. In any case, microorganisms in the lamina propria are either phagocytosed and eliminated by macrophages [29] or engulfed by DCs (along with B cells, both are considered the “professional” antigen presenting cells (APCs)) and are carried live to the mesenteric lymph nodes (Figure 1(c)). Recognition of infected apoptotic cells and bacteria results in the upregulation of interleukin 6 (IL-6), which drives the differentiation of proinflammatory T-helper-IL-17-producing (Th17) cells. Th17 cells primarily produce two main members of the IL-17 family, IL-17A and IL-17F which are involved in the recruitment, activation, and migration of neutrophils [30], with granulocytes playing an important role in bacterial clearance. Commensal-bearing DCs also induce the production of protective secretory immunoglobulin A (sIgA) in activated B cells (Figure 1(c)) [31], that is, plasma cells. This sIgA is then distributed across all mucosal surfaces through the recirculation of activated B and T cells. Commensal bacteria also directly promote the expression of factors involved in the induction of IgA⁺ B cells (Figure 1(c)) [32], their survival, and, interestingly, production of the more resistant form of sIgA, exerting its stability and antimicrobial properties [33]. Through this constant “priming” across several layers, microbiota maintains the immune system's readiness and reactivity, making it more capable of a quick and effective response when needed.

Specific populations of commensal bacteria, for example, *Bacteroides fragilis*, *Bifidobacterium infantis*, and *Clostridium* clusters IV and XIVa, also induce the so-called regulatory T cells (Tregs), a subset of forkhead box P3 positive (FoxP3⁺) CD4⁺ T-helper lymphocytes that maintain gut homeostasis by stimulating the production of anti-inflammatory cytokine

TABLE 1: Most frequently detected bacteria in GI tract and respiratory system of healthy volunteers or from healthy tissue samples. Results from different studies are presented by the taxa level in which they were originally detected, in order of decreasing abundance where possible. If the sampling, analysis method, or result was specific for a certain study, the reference was added adjacent to the corresponding information.

	Sample source Analysis method	Phylum	Order or family	Genus or species	Reference
GI tract	Faeces qPCR ^a , 16S sequencing ^b	Firmicutes (79.4% of sequences), Bacteroidetes (16.9%), Actinobacteria (2.5%), Proteobacteria (1%), and Verrucomicrobia (0.1%) ^b		<i>Faecalibacterium</i> , <i>Ruminococcus</i> , <i>Eubacterium</i> , <i>Dorea</i> , <i>Bacteroides</i> , <i>Alistipes</i> , and <i>Bifidobacterium</i> ^a	Tap et al. [11]
	Saliva 16S sequencing	Firmicutes, Proteobacteria, Actinobacteria, Fusobacteria, TM7, and Spirochaetes	Pasteurellaceae (5.8%), Enterococcaceae (2.6%), Veillonellaceae (2.0%), Burkholderiales (1.2%), and Lactobacillales (1.1%)	<i>Neisseria</i> and <i>Streptococcus</i> (70% of sequences), <i>Haemophilus</i> (9.2%), <i>Leptotrichia</i> (2.1%), <i>Actinomyces</i> (1.9%), <i>Abiotrophia</i> (1.8%), <i>Atopobium</i> (1.6%), <i>Gemella</i> (1.6%), and <i>Arthrobacter</i> (1.0%)	Lazarevic et al. [12]
Nose	Swab 16S sequencing	Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes, and Fusobacteria	Staphylococcaceae, Lachnospiraceae [13] Propionibacteriaceae, Corynebacteriaceae [14]		Charlson et al. [14] Lemon et al. [13]
Oropharynx	Swab 16S sequencing	Dominated by Firmicutes, Proteobacteria, and Bacteroidetes, and Fusobacteria, Actinobacteria, TM7, and SRI follow (oropharynx was richer and less variable than the nostril microbiota)	Streptococcaceae, Lachnospiraceae, unclassified group of Clostridia [13] Streptococcaceae, Veillonellaceae, Fusobacteriaceae, and Neisseriaceae [14]		Charlson et al. [14] Lemon et al. [13]
Esophagus	Biopsy 16S sequencing	Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and TM7		<i>Streptococcus</i> (<i>mitis</i> , <i>thermophilus</i> , <i>parasanguis</i>), <i>Prevotella</i> (<i>paullens</i>), <i>Veillonella</i> (<i>atypica</i> , <i>dispar</i>), <i>Rothia</i> (<i>mucliginosus</i>), <i>Megasphaera</i> (<i>micronucleiformis</i>), <i>Granulicatella</i> (<i>adiacens</i>), <i>Gemella</i> , TM7 <i>Actinomyces</i> (<i>odontolyticus</i>), <i>Bacteroides</i> , <i>Clostridium</i> , <i>Haemophilus</i> , and <i>Bulleidia</i> (<i>moorei</i>)	Pei et al. [15]
Lung	BAL ^a , brushing ^b 16S sequencing	Actinobacteria, Firmicutes, and Proteobacteria ^a [16] Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Fusobacteria ^a [7]	Streptococcaceae, Veillonellaceae, Prevotellaceae, Micrococcaceae, Neisseriaceae, Porphyromonadaceae, Lachnospiraceae, Actinomycetaceae, and Fusobacteriaceae ^a [14]	<i>Pseudomonas</i> , <i>Streptococcus</i> , <i>Prevotella</i> , <i>Fusobacterium</i> , <i>Haemophilus</i> , <i>Veillonella</i> , and <i>Porphyromonas</i> ^a [17] <i>Prevotella</i> , <i>Veillonella</i> , other <i>Firmicutes</i> , other <i>Bacteroidetes</i> , <i>Streptococcus</i> , <i>Haemophilus</i> , <i>Neisseria</i> , <i>Fusobacterium</i> , other <i>Actinobacteria</i> , <i>Staphylococcus</i> , other <i>Proteobacteria</i> , and <i>Corynebacterium</i> ^b [7]	Charlson et al. [14] Erb-Downward et al. [17] Hilty et al. [7] Pragman et al. [16]

BAL: bronchoalveolar lavage.

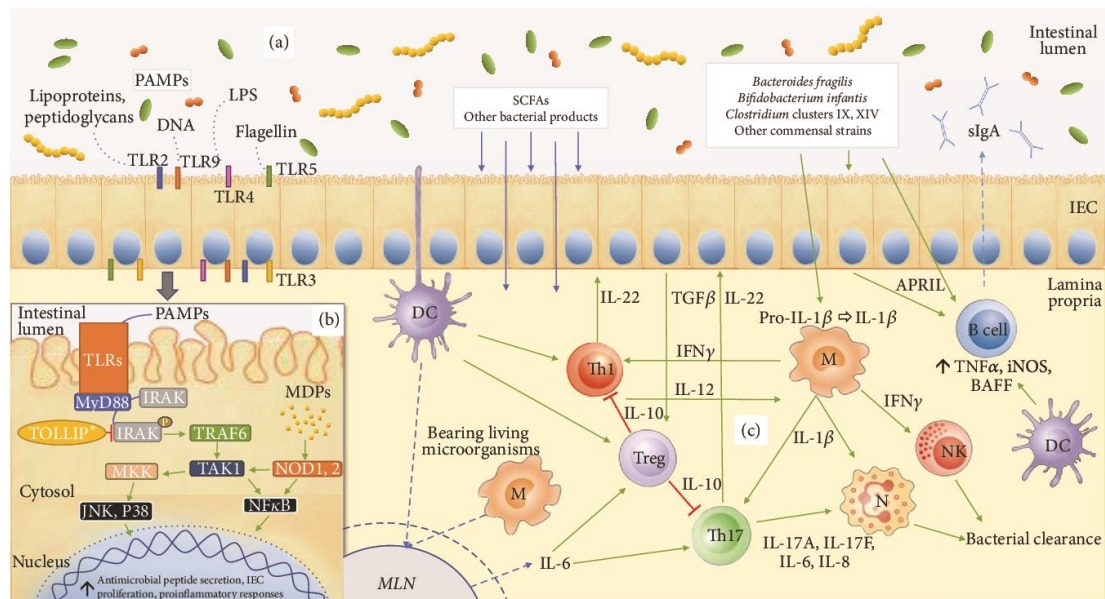


FIGURE 1: Interaction of the microbiota and intestinal mucosa. (a) Microorganisms in the intestine provide pathogen-associated molecular patterns (PAMPs) that serve as ligands for different Toll-like receptors (TLRs) on the luminal or basolateral surface of the intestinal epithelial cells (IECs). (b) TLRs stimulation activates a signalling cascade resulting in transcription factor activation and gene transcription, enhancing the cell barrier and further stimulating the immunological cells in the lamina propria. This cascade can be inhibited by toll-interacting protein (TOLLIP). *Inhibition seen only for TLR2 and TLR4 [23]. (c) Commensal bacteria and their derivatives (e.g., short-chain fatty acids (SCFAs)) directly stimulate IECs (b) or can be phagocytosed by DCs and macrophages in lamina propria and carried to mesenteric lymph nodes (MLN) where they prime naïve B and T cells to mature and differentiate. B cells become plasma cells and produce IgA that is secreted into the intestinal lumen (sIgA). T cells profile into Th17 and Th1, with proinflammatory tendency, activating additional effector cells as neutrophils, resulting in bacterial clearance. There is also differentiation to Treg cells having anti-inflammatory properties and controlling inflammation. APRIL: a proliferation-inducing ligand; BAFF: B cell-activating factor of the tumour necrosis family; DC: dendritic cell; IEC: intestinal epithelial cells; IFN: interferon; IL: interleukin; iNOS: inducible nitric oxide synthase; IRAK: interleukin receptor-associated kinase; JNK: c-Jun N-terminal kinases; LLN: lung lymph node; LPS: lipopolysaccharide; M: macrophage; MDPs: microbiota derived particles; MKK: mitogen-activated protein kinase kinase; MLN: mesenteric lymph node; MyD88: myeloid differentiation primary response gene 88; N: neutrophil; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; NK: natural killer cell; NOD: nucleotide-binding receptor; PAMPs: pathogen-associated molecular patterns; SCFAs: short-chain fatty acids; TAK-1: transforming growth factor beta-activated kinase; TGFβ: transforming growth factor beta; Th: T-helper cell; TLR: toll-like receptor; TNFα: tumour necrosis factor alpha; TOLLIP: toll-interacting protein; TRAF6: TNF receptor-associated factor 6; Treg: regulatory T cell.

IL-10 [34]. These cells serve as a kind of counterbalance to Th17 response, controlling the scope of its reaction and proinflammatory cytokine production. Thus, depletion of Tregs leads to the abnormal expansion of CD4⁺ Th cells, resulting in robust IL-17 and interferon gamma (IFNγ) responses in the colonic lamina propria, to produce intestinal inflammation.

To understand more precisely which commensal microbiota has this immunostimulatory effect, the well-known *Bifidobacterium* probiotic group was tested for its ability to induce the full maturation of human peripheral blood mononuclear cells (PBMCs) into DCs. Twelve *Bifidobacterium* strains descending from 4 probiotic species (*Bifidobacterium longum*, *B. breve*, *B. bifidum*, and *B. animalis* subsp. *lactis*) were tested, and each was induced to full maturation into DCs but using different Th preferences

(Th1 or Th17), depending on the type of *Bifidobacterium* strain with which they were incubated. Also, cell-free culture supernatants were poor inducers of maturation [35], leading us to conclude that live bacterial cells are necessary to induce efficient maturation and antigen presentation in DCs with this type of bacteria.

Likewise, introducing probiotic strains such as *Bifidobacterium lactis* into healthy elderly volunteers with fully developed immune systems resulted in a significant increase in the proportion of mononuclear leukocytes, the phagocytic capacity of mononuclear and polymorphonuclear phagocytes, and the tumoricidal activity of NK cells [36], highlighting the importance of this specific immune-boosting species whose presence directly impacts immune status.

Immunity and inflammation are not necessarily affected by bacterial cells but may be influenced by bacterial products.

Bacterial products that have a significant effect on overall host status surely included short-chain fatty acids (SCFAs), by-products of the microbial fermentation of dietary fibre. Among others, *Bacteroides*, *Bifidobacterium*, *Propionibacterium*, *Eubacterium*, *Lactobacillus*, *Clostridium*, *Roseburia*, and *Prevotella* are all remarkable SCFA producers [37]. Fermentation and SCFA production are thought to inhibit the growth of pathogenic organisms by reducing luminal pH [38]. The most abundant SCFAs are acetate, propionate (found mostly in the small and large intestines), and butyrate (found mostly in the caecum and colon), which are primarily derived from carbohydrates [39]. SCFAs have their specific receptors both on leukocytes and on endothelial cells. Known as two formerly orphan G protein-coupled receptors, GPR41 is found in a wide range of tissues including neutrophils, while GPR43 is shown to be highly expressed in immune cells [40]. GPR109a, the third receptor found in the colon epithelium and immune cells, is butyrate-specific and closely associated with the anti-inflammatory effect [41]. Butyrate is one of the most important SCFAs with members of the Firmicutes phylum as major butyrate producers, harbouring genes for the acetyl-CoA pathways. Other than as the main energy source for the intestinal epithelium and its role in barrier integrity [42, 43] following selective transport into the colon epithelium, butyrate manifests broad anti-inflammatory activities such as immune cell activation, proliferation, migration, adhesion, cytokine expression, and cancer cell apoptosis [26, 41]. These are mostly attributed to its function as a histone deacetylase (HDAC) inhibitor. HDAC inhibition influences the acetylation not only of histones (FoxP3 locus in Tregs, important for their maturation) [44], but also of major transcription factors such as nuclear factor kappa-light-chain-enhancer for activated B cells (NF- κ B) or signal transducer and activator of transcription 3 (Stat3) [45, 46], major proinflammatory pathway factors affecting the proinflammatory cytokine secretion profile in immune cells [47] and decreasing the proliferation and apoptosis of tumour cells [48].

As mentioned previously, changes to the gut microbiota are related to, for example, changes in diet, antibiotic administration, chemotherapy, and a person's general immune status. Whether with a transient or permanent effect, these changes often lead to dysbiosis, with an altered ratio of beneficial bacterial species (*Lactobacillus* sp., *Faecalibacterium prausnitzii*, etc.) and/or an overgrowth or population shift of other species [26]. In this event, expanded indigenous microorganisms (now potential opportunistic pathogens) may also produce DNA-damaging superoxide radicals and genotoxins in significant concentrations and induce innate immune mediated proinflammatory pathways [49], directly damaging cells and promoting malignant transformation by inducing chromosomal and microsatellite instability, CpG island methylation, epigenetic alterations, and posttranslational modifications, which weaken the immune response and increase the risk of cancer [50].

Apart from the direct pathological effect, an absence of the appropriate microbial composition in the immune system's early development has more far-reaching effects. This is evident from studies on mice reared in germ-free (GF)

conditions. These animals have impaired GI-driven immune development, characterized by smaller Peyer's patches, fewer CD8 $\alpha\beta$ intraepithelial lymphocytes, underdeveloped isolated lymphoid follicles, a lack of primed T cells, lower levels and impaired production of mucosal IgA antibodies, and active IL-10-mediated inflammatory hyporesponsiveness [51, 52]. Also, mice with colitis-associated cancer (CAC) that lacked microbiota were unable to process pro-IL-1 β and pro-IL-18 (interleukins in this case necessary for a desirable inflammatory reaction) into their mature forms, resulting in increased tumour burden [53]. That said, we can see that the composition of "healthy" microbiota is crucial to the proper development of the immune system's basic structures. Unless fully developed, their ability to exchange information and their reactions to the "outside" world are compromised. This state could be characterized as similar to anergy: the signal is present but the immune system does not respond, as exemplified in the case of CAC mice.

3. Lung Microbiota

The human respiratory tract is the primary and continuous entry portal for numerous microorganisms and particles, such as viruses, bacteria, or fungi. These are primarily airborne but can also be transferred through saliva. Below the vocal cords, the human airways harbour bacteria and other microbes in rich surroundings [54] that are distinct in composition from the microbiota seen at other sites (in the nasal and oral cavity, gut, skin, and vagina). Despite being less populated compared to the GI tract, the lung microbiota includes a range of microorganisms, mostly seen through the use of bronchoalveolar lavage (BAL) fluid, or tissue samples. Since lung microbiota exploration has rather young and nonuniform protocols, it is crucial to bear in mind that the type of sample (lavage, tissue), sampling method, and possibility of cross-contamination during sampling between distinct parts of the airway influence final results [19, 55]. Therefore, due to the paucity of overall studies in this field, one must be careful to take detailed methodology and its possible advantages and disadvantages into account. Studies that analyse lung tissue acquired through sterile surgical explant have also been carried out, and they all report that the lower respiratory tract contains a microbiome that is distinct from, but related to, that of the upper airways [56].

In 2014 Dickson et al. [57] proposed an adapted island model for lung biogeography, suggesting that more distal lung bacterial communities are less rich and more dissimilar to their upper respiratory tract source community. Here, microbial composition is determined by the rate of microbial immigration into the airways, their rate of elimination (e.g., by coughing or immune defences), and the rate at which different community members are reproduced [58]. Temperature, oxygen tension, pH, nutrient density, local anatomy, and host defence are spatially heterogeneous across the airways and the lungs, all of which affect local microbiological growth conditions.

Starting with the upper respiratory airways, the nostril is dominated by Firmicutes and Actinobacteria; Firmicutes, Proteobacteria, and Bacteroidetes are prevalent in the oropharynx [13]. In the lung the most common phyla consistently observed are Bacteroidetes, Firmicutes, and Proteobacteria. The nasal microbiota seems to more closely resemble that of the skin than that of the lungs and contributes little to lung communities [59]. The coming years are likely to bring the development of new methods able to minimize cross-contamination (as seen for endoscopy [60]), which is the biggest problem in lower respiratory tract sampling that, along with metagenomic analysis (of both cultivable and noncultivable bacteria), will yield more precise results in terms of the bacterial population found at certain airway depths. An overview of different studies investigating the composition of the respiratory system microbiota is presented in Table 1.

The ecological determinants of the lung microbiota (immigration, elimination, and regional growth conditions) change during acute and chronic lung disease, as seen in chronic obstructive pulmonary disease (COPD) (often a precancerous inflammatory state) and lung cancer (Table 2) [7, 19]. Whether the observed dysbiosis is a cause, consequence, or simply a coevolving factor still needs to be elucidated, but its likely role will be individually connected to the pathology and its aetiology. However, it is known that smouldering inflammation (caused by lung injury, pathogen colonization, or intrinsic factors) is often a common starting point that leads to subsequent cancer development [61].

The microbial factors that may be responsible for lung cancer development are still not well known, unlike the many genetic predispositions and mutations that underlie the different types of lung cancer [62]. This is why, for now, it is possible to correlate the nongenetic development of lung cancer with the well-characterized development of COPD, a chronic inflammatory state where initial lung injury, whatever its cause, creates an opening for microbial dysbiosis and colonization, thus worsening the overall condition and often leading to the cancer state.

Inflammation of the lung is associated with a loss of epithelial integrity and results in the “leakage” of serum proteins into the airways [63]. Formyl peptides and cleavage products of bacterial or mitochondrial proteins, as well as other bacterial products, serve as powerful chemoattractants for both the neutrophils and monocytes that emigrate from alveoli [64]. Although essential in pathogen clearance and having a tumoricidal effect [65], neutrophil influx and degranulation in the airways and lung parenchyma contribute to chronic inflammation, parenchymal lung damage, and progressive small airway obstruction, due to the loss of alveolar attachments and lung elasticity [66], as explained by Sethi’s vicious circle hypothesis [67]. *In vitro*, their enzymes, serine proteinases (elastase, cathepsin G, and proteinase 3), and defensins markedly affect the integrity of the epithelial layer, decreasing the frequency of the ciliary beat, increasing mucus secretion, and inducing the synthesis of epithelium-derived mediators such as neutrophil chemoattractant chemokine IL-8 from respiratory epithelial cells [68].

Obstruction of the lumina with mucus introduces pockets of increased temperature and decreased oxygen tension, selectively favouring the growth of well-known disease-associated microbes [69, 70]. This dysbiotic shift can be characterized by a move away from the Bacteroidetes phylum, often to Proteobacteria (e.g., *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Moraxella catarrhalis*) [17, 20], and sometimes to Firmicutes (e.g., *Streptococcus pneumoniae* and *Staphylococcus aureus*) [19]. The same growth effect was observed with a generation of intra-alveolar catecholamines and inflammatory cytokines [71]. If airway colonization becomes persistent, it further promotes chronic inflammatory response, affecting the elastase-antielastase balance in the lung, which was shown to vary 80-fold with changes to the airway bacterial load [72]. A higher bacterial load consequently induces higher overall IL-8 and other blood circulating inflammatory cytokine levels, greater inflammation, oxidative stress, and greater forced expiration volume (FEV) decline [73].

Lung microbiota was also shown to vary according to clinical endpoints. In nonmalignant lung tissue from advanced stage cancer, alpha diversity had increased, while it had decreased in the tumour lung tissue. Also, the interaction between the upper and lower airways involving the microbial population present in lung cancer is clearly shown in the study carried out by Yan et al. [21]. The study showed that there is a high degree of specificity in patients with either small cell carcinoma (SCC) or adenocarcinoma (AC), compared to controls based on bacteria isolated from saliva (Table 2).

As mentioned above, IL-6 and IL-8 are cytokines that become elevated during inflammatory stress. They are involved in tumorigenesis by acting directly on lung epithelial cells to stimulate the NF- κ B-1 pathway [74]. Additionally, IL-6 and IL-8 are expressed by premalignant or senescent lung cancer cells [75]. They may act in an autocrine and/or paracrine fashion to stimulate cancer cell proliferation [76], migration, and invasion [77]. In bronchoalveolar carcinoma, tumour cells were a main source of IL-8 and the presence of an increased number of neutrophils in BAL fluid was correlated with the IL-8 level in BAL and associated with a poor outcome [78]. In a case study carried out by Pine et al. [79], increased levels of both serum IL-6 and IL-8 were associated with lung cancer, but only the IL-8 level was associated with lung cancer risk several years prior to diagnosis.

To summarize, the appearance of dysbiosis or malignancy is likely the product of a dynamic interaction between various immune, microbial, and environmental factors. At least one of these acts as an initiator but others often readily follow. This is why it remains difficult to reach any conclusions regarding the true aetiology of disease and what might be the best intervention and, more importantly, prevention approach.

4. A Bidirectional Concept of the Gut-Lung Axis

Recently, we have reached a greater understanding of microbial influence on the complex and interconnected axis between gut and lung. This stems from the simple fact

TABLE 2: Most frequently detected bacteria in the lung of patients suffering from their respective diseases. Results from different studies were presented by the taxa level in which they were originally detected, in order of decreasing abundance where possible. If the sampling, analysis method, or result was specific for a certain study, the reference was added adjacent to the corresponding information.

Disease	Sample source Analysis method	Phylum	Order or family	Genus or species	Reference
COPD	BAL ^a , sputum ^b , lung tissue ^c 16S sequencing ^d , qPCR/DGGE ^e , bacterial culture ^f	Proteobacteria (44%) and Firmicutes (16%) followed by Actinobacteria (13%), with Bacteroidetes, Fusobacteria, Tenericutes, SRI incertae, TM7, and Synergistetes identified in lower proportions (<3%) ^{b,d} [18]		<i>Haemophilus influenzae</i> , <i>Pseudomonas aeruginosa</i> , <i>Moraxella</i> <i>catarrhalis</i> , and <i>Streptococcus</i> <i>pneumoniae</i> ^{b,c} [18]	Erb-Downward et al. [17] Garcia-Núñez et al. [18] Hilty et al. [7]
		Increase in Proteobacteria (sometimes Firmicutes), decrease in Bacteroidetes and Firmicutes ^{c,d} [19]		Significant increases of <i>Streptococcus pneumoniae</i> , <i>Klebsiella</i> <i>pneumoniae</i> , and <i>Pseudomonas</i> <i>aeruginosa</i> ^{b,c} [20]	Sze et al. [19] Wu et al. [20]
Lung cancer	Sputum ^a , saliva ^b anaerobic culture ^c , 16S sequencing ^d	In moderate COPD mostly Actinobacteria and Proteobacteria In severe COPD mostly Actinobacteria and Firmicutes ^{a,d} [16]		<i>Tropheryma</i> , <i>Actinomyces</i> , <i>Peptostreptococcus</i> , <i>Serratia</i> , <i>Capnocytophaga</i> , <i>Leptotrichia</i> , <i>Kingella</i> , and <i>Dysgonomonas</i> ^{a,d} [16]	Pragman et al. [16]
		Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, and Fusobacteria ^{b,d} [7]	Flavobacteriales, Burkholderiales, Campylobacteriales, Spirochaetales (more abundant), and Bacteroidales (less abundant) ^{b,d} [21]	<i>Prevotella</i> , <i>Haemophilus</i> , <i>Streptococcus</i> , <i>Neisseria</i> , other <i>Proteobacteria</i> , <i>Veillonella</i> , other <i>Firmicutes</i> , other <i>Bacteroidetes</i> , <i>Fusobacterium</i> , other <i>Actinobacteria</i> , and <i>Staphylococcus</i> ^{b,d} [7]	
		Veillonellaceae significantly overexpressed whereas Lachnospiraceae underexpressed ^{b,d} [21]	<i>Actinomyces</i> spp., <i>Peptostreptococcus</i> spp., <i>Eubacterium lentum</i> , <i>Veillonella parvula</i> , <i>Prevotella</i> spp., <i>Bacteroides</i> spp., <i>Lactobacillus</i> <i>jensenii</i> ^{a,c} [22]	Rybojad et al. [22] Yan et al. [21]	
			<i>Capnocytophaga</i> , <i>Selenomonas</i> , and <i>Veillonella</i> were found to be more abundant in both SCC and AC ^{b,d} [21]		

COPD: chronic obstructive pulmonary disease; BAL: bronchoalveolar lavage; DGGE: PCR-denaturing gradient gel electrophoresis; SCC: small cell carcinoma; AC: adenocarcinoma.

that ingested microorganisms can access both sites—from gastrointestinal tract microbiota that enters the lung through aspiration [80] to the more “internal” influence, which shows improved lung function and pathogen clearance following the transplantation of faecal microbiota [6].

This interaction can be mediated in different ways—by the microbiota and its products or via immune cells (Figure 2). According to the “gut-lymph” theory of Samuelson et al. [26], there are sufficient macrophages and other immune cells in the intestinal submucosa or the mesenteric lymph nodes that contain a majority of translocating bacteria. Surviving bacteria, cell wall fragments, or the protein parts of dead bacteria escaping with the cytokines and chemokines produced in the gut travel along the mesenteric lymphatic system to the cisterna chyli and subsequently enter the systemic circulation. Access to pulmonary circulation may lead to DC and macrophage activation as well as the priming of T cells and their differentiation.

Another way to influence the pulmonary region is through the migration of immunological cells. As previously mentioned, translocated microorganisms and their parts within the lamina propria are transferred to the mesenteric lymph nodes by antigen presenting cells (APCs) and used for priming naïve B and T cells. Activated B cells capable of producing antigen-specific immunoglobulins, that is, plasma cells, will not only produce immunoglobulins in situ, but will reach draining lymph nodes and other mucosal tissues, thereby spreading immunological “information.” The constitutive entry of antigen at steady state stimulates inflammation—conversion of pro-IL-1 β and pro-IL-18 into active form; in other words it switches off our innate ability to produce IL-10 and other anti-inflammatory molecules leading to DC migration to local lymph nodes and the priming and differentiation of T cells. The latter can subsequently migrate out of the gut-associated lymphatic tissue (GALT) and reach both mucosal and peripheral nonmucosal tissues, including the bronchial epithelium, thus modifying the immunological response which is dependent on the induced cell profile (to Th1, Th2, etc.) [81, 82] and improving the immunological response against pulmonary pathogens [83].

Although this theory explains the unilateral interaction, it is reasonable to speculate that this axis works in precisely the same way when it originates in the lung mucosa and lung lymph nodes (Figure 2). Further, lung DCs *in vitro* have the option to imprint the expression of gut-homing integrin $\alpha 4\beta 7$ and CCR9 (lung-homing integrin is CCR4 [84]) on cocultured T cells *in vitro* and on adoptively transferred cells *in vivo*, which guides their migration to the GI tract [85].

4.1. Influence of the Gut Microbiota on the Lung. The composition of “healthy,” or rather balanced, gut microbiota is shown to have a serious influence on the effectiveness of lung immunity. GF mice, devoid of their intestinal microbiota during the development of their immune system, show impaired pathogen clearance in the lung, which results in their growth and dissemination [52]. At this stage, it is also important to consider that the lungs of these mice are also germ-free [86], devoid of all microbiota that might normally play a role in stimulating lung immunity. Modified alveolar architecture

also results, and thus both factors modify the response to pathogen infection. The same observations were made as with the infection of GF animals, when wild phenotypes were treated with antibiotics, thus disturbing the intestinal homeostasis, after they had been challenged with bacterial or viral microorganisms [87, 88]. If animals had been boosted with lipopolysaccharide (LPS) following antibiotic treatment, they were better able to cope with the lung infection and consequently had reduced mortality. Population studies followed these findings with respect to the importance of preserving gut microbiota, showing that increased use of penicillins, cephalosporins, macrolides, and quinolones correlated with an increased risk of lung cancer in humans [89]. Here we can speculate that various antibiotic treatments eradicate the bacterial populations required for effectively priming T lymphocytes with antitumour properties, while at the same time making room for other opportunistic pathogens to colonize both the gut and the lung.

Interestingly, modified gut microbiota not necessarily characterized as dysbiotic may also influence immune response efficiency, as seen in obese mice. These mice had an impaired expression of cytokines in their lungs (IFN α , IFN β , IL-6, and TNF α) and significantly decreased mRNA of IFN γ , interleukin 2 receptor subunit beta (IL-2RB), and perforin 1 (Prfl). All the criteria were improved following daily supplementation with a probiotic strain of *Lactobacillus gasseri* [90].

Nutrition may also impact microbial development and the composition of our respiratory tract microbiota [91]. A high-fibre diet in mice has been shown to increase SCFA circulating blood levels (but no traces in the lung itself) and has been shown to be responsible for increased protection against allergic inflammation in the lung (reduced inflammatory cell infiltration), followed by a change in the intestinal and, to a lesser extent, the airway microbiota [92].

The above-mentioned findings clearly show how important the overall composition of the intestinal microbiota is for a productive immunological response in the lung. Lack of an appropriate stimulus during the developmental phase, as during infection, will disable a quick and effective immune reaction, resulting in pathogenic colonization, increased susceptibility to infection, damage, the possible development of cancer, and increased mortality. At the same time, just one single strain, bacterial part, or product can turn the tables and provide the boost needed to stimulate the correct immune response.

4.2. Influence of the Lung Microbiota on the Gut. Unlike the local and systemic influence of intestinal microbiota, the influence of lung microbiota and its products and their circulation is yet to be properly assessed. One study reported that nonabsorbable tracer deposited into the nasal cavity of mice can be found in the GI tract a short time later [93]. Also, Sze et al. [54] showed that, in mice, even acute exposure to a single dose of intratracheal LPS disrupts the airway microbiota, leading to translocation of these bacteria into the bloodstream. Within 24 hours the caecal microbiota is also disturbed, leading to a sharp increase in the total bacterial load. It still remains unclear as to whether this effect is

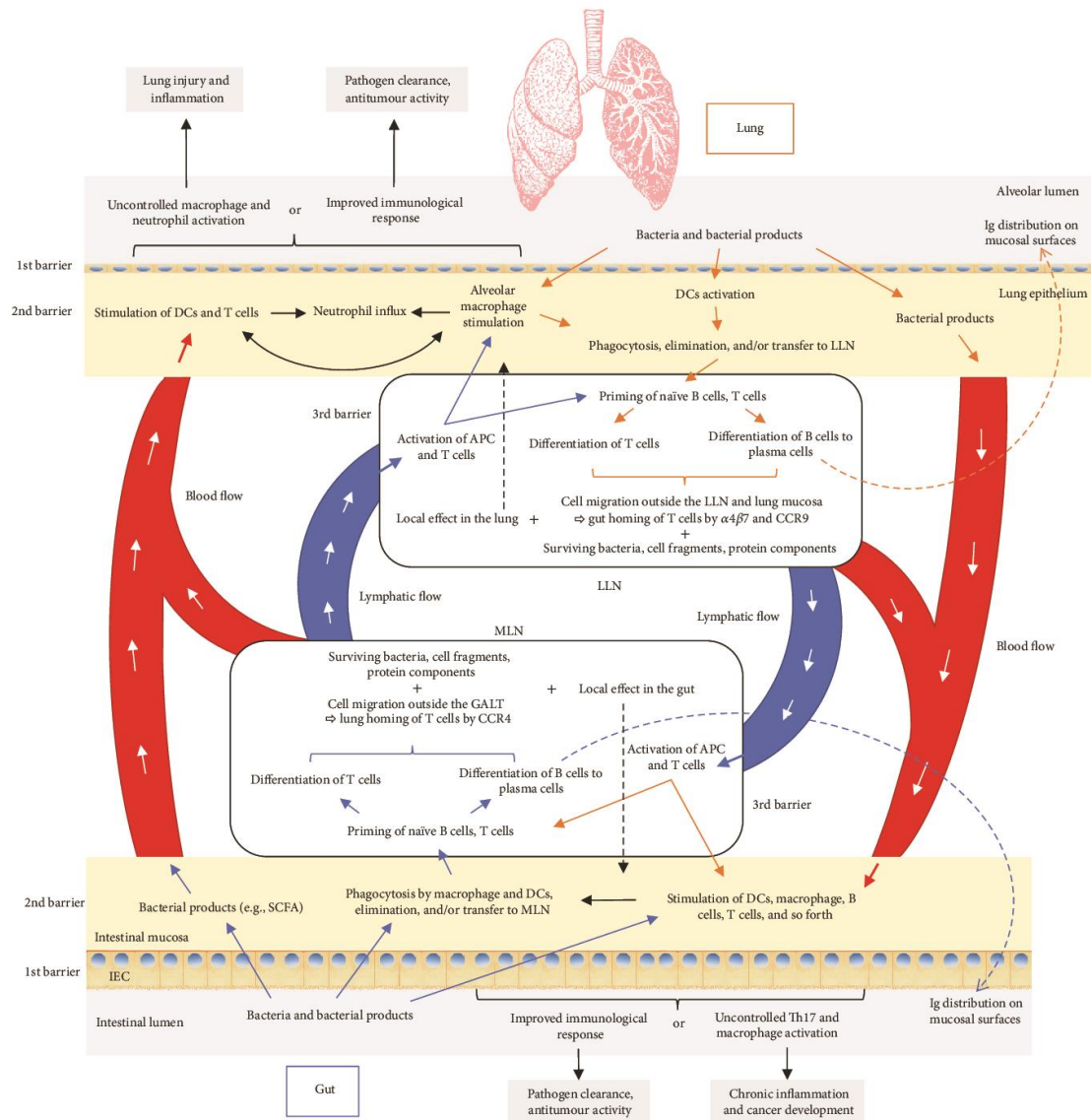


FIGURE 2: Proposed pathways of the gut-lung interaction. Microbiota and its products that enter intestinal mucosa (blue arrows) are phagocytosed and transferred to mesenteric lymph nodes (MLN) by antigen presenting cells (APC), where they stimulate priming of the T and B cells. Once activated, with the expression of proper homing receptors, these cells can migrate back to the original site (intestinal mucosa) (black dashed arrow) or to distal locations such as the lung epithelium and lung nodes through lymphatic and blood circulation. There, they can directly act on their target or continue to stimulate other immune cells. On the other hand, bacterial products from the intestinal mucosa or surviving bacteria can also reach the lung by blood or lymphatics to stimulate the immune system in the same way as they would have done in the intestinal tract. Depending on the tissue prestimulation, type of stimulus, and local and general immunological status, the result can be positive, as effective bacterial clearance or antitumour activity, or overinflammatory response, promoting further tissue damage, pathogen colonization, and tumour progression. The same schema is proposed in the other sense, beginning with the lung mucosa and finishing with distal effect on the gut. Although not known for the moment, there is also a possibility that bacterial products of the lung microbiota can exert their effect in the intestinal mucosa, being delivered in the same way as explained above. APC: antigen presenting cell; DC: dendritic cell; GALT: gut-associated lymphatic tissue; IEC: intestinal epithelial cell; LLN: lung lymph node; MLN: mesenteric lymph node; SCFA: short-chain fatty acid. Colour legend (borders/arrows): blue: influence of gut on lung; orange: influence of lung on gut; black: mutual influence.

due to the direct interaction of translocated pulmonary and residential intestinal microbiota via immune cell or cytokine mediation or to microbial products that reach the gut.

4.3. Microbiota and Cancer via the Immune System. Due to a number of genetic alterations resulting in the loss of normal cellular regulatory processes, cancer cells express neoantigens that are tumour-specific and distinguish tumour cells from healthy cells. The importance of the gut microbiota in anticancer response has been described by Chen and Mellman [94] through the concept of a cancer-immunity cycle. The cancer-immunity cycle starts with the capture of neoantigens from cancer cells by DCs. For an anticancer response to take place, this must be accompanied by another signal, such as proinflammatory cytokines, factors released by dying cancer cells or by gut microbiota components. The goal here is to reduce peripheral tolerance to tumour antigens. Following processing, DCs present captured neoantigens to T cells, thus resulting in their priming and activation to create effector T cells against cancer-specific antigens. At this point, the balance between T effector and T regulatory cells is crucial in determining the nature of the immune response. The now activated T effector cells travel to the tumour site, invade the tumour bed, and, by recognizing specific tumour antigens, bind and kill cancer cells. Problems arise when tumour antigens are not detected, meaning that DCs and T cells treat antigens as “self” rather than foreign. In this case, a Treg response rather than an effector response results. Homing of T cells to tumour may not be correct either. T cells may be inhibited from infiltrating the tumour, or (more importantly) factors in the tumour microenvironment may suppress any effector cells that are produced. There are two main negative regulators of T cell responses: checkpoints in lymphoid organs (CTLA-4) and immunostats within the tumour beds (PD-L1:PD-1). Programmed cell death ligand 1 (PD-L1) is a molecule expressed on tumour cells or on tumour-infiltrating immune cells, which binds programmed cell death protein 1 (PD-1) expressed on effector CD8⁺ T lymphocytes, blocking the secretion or production of the cytotoxic mediators needed to kill tumour cells within the tumour beds. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), expressed on Tregs, acts as the major negative regulator of the priming and activation of effector CD8⁺ T cells inside the lymphoid organs, by binding its CD80 and CD86 ligands on APCs. The presence of these suppressive factors explains the limited activity of previous immune-based therapies. The goal of current cancer immunotherapy, using anti-PD-L1:PD-1 and anti-CTLA-4 antibodies, is to initiate or reinstate a self-sustaining cycle of cancer immunity, enabling it to amplify and propagate without creating an unrestrained response.

To create a higher response to neoantigens, the immune system's peripheral tolerance must be reduced. It is already known that the commensal microbiota induces the generation of CD4⁺ T cells against their own antigens [95] thereby limiting the systemic dissemination of commensal bacteria [96]. The same antigen cross-reactivity effect, or superantigen-driven response, accounts for T cell-dependent tumour regression. As suggested by Viaud et al. [47] and based on recent studies by Iida et al. [97] and Viaud et al. [98]

in mice, Th17 cells and memory Th1 cells elicited against commensal bacteria might preferentially accumulate in an inflammatory tumour microenvironment, already primed by bacterial products or ligands for PRRs. Based on these studies, Zitvogel et al. [99] explain the long-range effect of microbiota through two signal hypotheses. Signal 1 hypothesis suggests a phenomenon of antigen mimicry or cross-reactivity. That is, certain microbial antigens from the bacterial species that pass the intestinal barrier and are used for T cell priming could closely resemble tumour antigens, thus promoting better immune system reactivity and antitumour response, that is, immunosurveillance. In signal 2 hypothesis, by interacting with PRRs after passing the intestinal barrier, microbiota can stimulate the production of a diverse palette of cytokines and interferons and determine whether it will elicit a proinflammatory, immunostimulatory, or immunosuppressive response. Also, there is some evidence that commensal-specific Tregs are capable of switching to effector inflammatory Th17 cells after sensing the disruption of the mucosal barrier. Because microbial products, metabolites, effector cells, and cytokines are able to travel, this stimulation is not necessarily confined to just the gut.

Observing these effects, it is interesting to speculate that at least a transient disruption of intestinal barrier functions and microbiota translocation is a primary factor in shaping the relationship between the gut microbiome, the immune system, and cancer.

4.4. Probiotics and the Lung. Probiotics, best known in nutritional therapy, are defined as “live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host” [100]. In the intestine they mainly refer to the genera *Lactobacillus* and *Bifidobacterium* and include many different strains such as *L. paracasei*, *L. rhamnosus*, *L. acidophilus*, *L. johnsonii*, *L. fermentum*, *L. reuteri*, *L. plantarum*, *B. longum*, *B. breve*, *B. bifidum*, and *B. animalis* subsp. *lactis*. The same genus does not necessarily involve the same characteristics, due to the great genomic differences between species and also within the different strains of the same species [101].

Although the first evidence of probiotic influence on lung cancer was seen in 1985 [102], probiotics only recently reemerged in the field of lung oncology as a possible new therapy and is already showing highly promising results. Using different mouse lung cancer models, conventional therapies (a combination of platinum-based agents with paclitaxel, gemcitabine, vinorelbine, or docetaxel, which all have high toxicity [103]) were combined with specific probiotic strains or, conversely, the antibiotic eradication of microbiota, to assess the effect of chemotherapy.

In a lung adenocarcinoma viral model, when vancomycin was used to eradicate Gram-positive bacteria it compromised the efficacy of cyclophosphamide- (CTX-) based chemotherapy and correlated with a reduced intratumoural CD8⁺ T effector/FoxP3⁺ regulatory T cell ratio [104]. Likewise, in mice treated with cisplatin combined with an antibiotic cocktail of vancomycin, ampicillin, and neomycin, tumour size was larger than that found in mice receiving a single treatment of cisplatin [105]. Taking these examples, we can

readily conclude that the presence of conventional intact microbiota is crucial for effective chemotherapy.

On the other hand, feeding mice orally with *L. acidophilus* (Lewis lung cancer model) treated with cisplatin decreases the size of tumours and improves the survival rate. Enhanced antitumour response is also achieved through upregulation of IFN γ , granzyme B (GzmB), and Prfl expression [105] following probiotic supplementation.

Recently, there was considerable interest in evaluating the role of gut microbiota in lung cancer therapy using immune checkpoint inhibitors. One of the first studies of this principle was done using a mouse melanoma model but is readily applicable to other cancer types, as shown in the study. Here, oral administration of a *Bifidobacterium* cocktail (*B. bifidum*, *B. longum*, *B. lactis*, and *B. breve*) on its own improved tumour control to the same degree as PD-L1-specific antibody therapy (checkpoint blockade) [106]. When the two treatments were combined, it virtually abolished tumour outgrowth. Improvement was seen in immune responses upstream of T cells, at the level of host DCs. Their augmented function enhanced CD8⁺ T cell priming and accumulation in the tumour microenvironment. The percentage of MHC II^{hi} DCs was also increased. With *Bifidobacterium* treatment, 760 genes were upregulated, including cytokine-cytokine receptor interaction, CD8⁺ T cell activation and costimulation, DC maturation, antigen processing and cross presentation, the chemokine-mediated recruitment of immune cells to the tumour microenvironment, and type I interferon signalling.

Vétizou et al. [107] reported that the oral feeding of GF mice with *Bacteroides fragilis* induced Th1 immune responses in tumour-draining lymph nodes and promoted the maturation of intratumoural DCs. This was, as the authors suggest, due to the cross-reactivity of the bacterial and tumour epitopes, which led to the restoration of the therapeutic response of GF tumour bearers to CTLA-4 antibody treatment. Antibody therapies such as this prevent inactivation of the CD8⁺ T cells by binding to the CTLA-4. Daillère et al. [108] noted that *Enterococcus hirae* and *Barnesiella intestinihominis* specific-memory Th1 cell immune responses selectively predicted longer progression-free survival in advanced lung cancer patients treated with chemoimmunotherapy. Both strains represent valuable “oncomicrobiotics” improving the efficacy of the most common alkylating immunomodulatory compound.

To summarize, as scientists delve deeper, the beneficial effects of probiotics on the immune system continue to emerge. As seen, certain strains have the power and ability to stimulate antitumour response or to simply stimulate the immune system to show lower tolerance, thus promoting higher reactivity and tumour eradication. The future objective is to find the optimum probiotic cocktail that may one day completely substitute conventional therapies, thereby obtaining equal or better success and lowering toxicity, one of the biggest problems in cancer treatment.

5. Conclusion

The importance of the gut microbiota and its composition has long been recognized, for digestion as well as for overall

wellbeing. Recently, the presence of the lung microbiota and the role it plays, in health and in disease, have been receiving attention. The lung and gut microbiota, both continually reseeded through interaction with the environment, modulate our local and systemic immunity. More than simply two distinct microbiota, they are now seen as functioning in dialogue, altering previous ideas of airway sterility and the existence of a “barrier” between the two compartments, due to their perceived distance or functional differences. By providing stimulating signals through its epitopes or products (such as SCFA butyrate), the gut microbiota directly enhances the intestinal barrier. Likewise, it stimulates the priming and maturation of T and B cells, ensuring improved microbial clearance and mucosal protection through antibodies. This effect is not only retained in the intestinal system but is spread along other mucosal surfaces by means of lymphatic and blood circulation, influencing distal site immune response. So, even though the antigen was introduced in the gut, an immunological response can also be elicited in the lung, although there was no direct prior contact with the antigen, and vice versa. Bacteria and their products that go through the first immunological barrier also reach distal sites through the lymphatic system and blood and modulate the immune response at the remote site. The site where the first encounter between the immune system and microbial antigens took place is also important, since it influences reactivity and the influx of these cells into other tissues. Applying prebiotics to target a specific microbial group could be a good way to restore “healthy” microbial composition, which will consequently increase intestinal barrier function and stimulate the immune system. The relevance of natural microbial support in chemotherapy effectiveness or replacement has already been demonstrated. In future, further discoveries will surely be made in this new and exciting area of research, adding to the complexity of, but also clarifying the reasons behind, this axis; opening ideas to new or enhanced therapies based on the natural behaviour of the organism; increasing longevity; and decreasing therapeutic side-effects or the effects of disease itself.

Conflicts of Interest

The authors hereby declare that no conflicts of interest exist with respect to the publication of this article.

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Annex 3

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Research Article

Study of the Effects of Betaine and/or C-Phycocyanin on the Growth of Lung Cancer A549 Cells *In Vitro* and *In Vivo*

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We investigated the effects of betaine, C-phycoyanin (C-PC), and their combined use on the growth of A549 lung cancer both *in vitro* and *in vivo*. When cells were cocultured with betaine and C-PC, an up to 60% decrease in viability was observed which is significant compared to betaine (50%) or C-PC treatment alone (no decrease). Combined treatment reduced the stimulation of NF- κ B expression by TNF- α and increased the amount of the proapoptotic p38 MAPK. Interestingly, combined treatment induced a cell cycle arrest in G₂/M phase for ~60% of cells. *In vivo* studies were performed in pathogen-free male nude rats injected with A549 cells in their right flank. Their daily food was supplemented with either betaine, C-PC, both, or neither. Compared to the control group, tumour weights and volumes were significantly reduced in either betaine- or C-PC-treated groups and no additional decrease was obtained with the combined treatment. This data indicates that C-PC and betaine alone may efficiently inhibit tumour growth in rats. The synergistic activity of betaine and C-PC on A549 cells growth observed *in vitro* remains to be further confirmed *in vivo*. The reason behind the nature of their interaction is yet to be sought.

1. Introduction

Lung cancer causes 28% of global cancer-related deaths and 15% of all diagnosed cancers [1]. It can be subdivided into two broad categories, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), the latter being the most common type [2]. The treatment of NSCLC includes surgery, radiotherapy, tyrosine kinase inhibitors, immunotherapy, and platinum-based chemotherapy [3]. With respect to chemotherapy, it appears that natural plant products play an important role, making a considerable contribution to approximately 60% of available chemotherapeutic cancer drugs [4].

The development and progression of cancer involve abnormal changes in DNA methylation which lead to the activation of certain protooncogenes, such as c-Myc, as well

as to the inactivation of certain tumour suppressors, such as p16 [5]. It seems that certain dietary components affect the process of carcinogenesis, through DNA methylation. In fact, it has been shown that folate, as methyl group donors, and riboflavin, vitamin B₆, and vitamin B₁₂, as cofactors in one-carbon metabolism, are associated with DNA synthesis and methylation and, hence, may play a role in carcinogenesis and cancer risk; DNA global hypomethylation is associated with lung cancer [6, 7]. Betaine (B) is another major methyl donor [8], which maintains normal DNA methylation patterns [9]. It is naturally found in a variety of food sources including sugar beet, wheat bran, spinach, shrimps, and many others [10]. Humans also obtain betaine from dietary choline, as the liver and kidney are able to oxidize choline into betaine in a two-step enzyme-dependent reaction [10, 11]. Betaine

spectrophotometer (Victor 3V Multilabel Plate Reader, Perkin-Elmer).

2.1.5. Western Blot. Cells were washed three times for 5 min with cold PBS; 50 μL of RIPA buffer (Sigma-Aldrich) with phosphatase and protease inhibitor cocktail (Sigma-Aldrich) was added to each well. Plates were incubated for 30 min at $+4^\circ\text{C}$ with orbital shaking. The cells were scrubbed, and two well contents were pooled and centrifuged (10 min, $3000\times g$, 4°C). Supernatants were stored at -20°C . On the day of analysis the samples were thawed; approximately 20 μg of each sample (protein quantification kit—BC assays, Interchim, Montluçon Cedex, France) was denatured with Laemmli 5x (10 min, 85°C), loaded on the 12% polyacrylamide gel, and migrated at 130 V (SDS-PAGE) (Mini-PROTEAN® Tetra Cell Systems, Bio-Rad). The transfer to nitrocellulose membrane was done for 80 min under 90 V (Bio-Rad PowerPac HC Power). Following transfer, the membranes were washed once in TBST (tris-buffered saline with Tween 20) and blocked for 1 h in 5% solution of bovine serum albumin (BSA) (Sigma) in TBST. The membranes were washed in TBST three times for 5 min and incubated with agitation overnight at 4°C with primary antibody diluted in 5% BSA solution. After incubation, membranes were washed 3 times for 5 min in TBST and incubated with horseradish peroxidase- (HRP-) coupled secondary antibody in 1% solution of dehydrated milk in TBST for 1 h at room temperature. The membranes were then washed three times for 5 min in TBST and two times in TBS. 3 mL of ECL™ Prime Western Blotting System (Sigma-Aldrich) was added to the membranes and incubated for 5 min. The blots were visualised by ChemiDoc™ XRS+ System with Image Lab™ Software (Bio-Rad). After visualisation of p38 MAPK, the membranes were incubated twice for 10 min with in-house dehybridization buffer, twice for 10 min with PBS, and twice for 5 min with TBST, and, after, the protocol was restarted from the blocking of the membrane for the final detection of the β -actin (incubation with the primary antibody was during 6 h on $+4^\circ\text{C}$).

The actin band was detected on the same membrane as the p38 MAPK. The signal quantification was done using Image J software. The primary antibodies used were p38 MAPK rabbit anti-human polyclonal antibody (ADI-KAS-MA009-E, Enzo Life Sciences) 1:500 and beta-actin rabbit anti-human polyclonal antibody (PA1-183, Thermo Scientific) 1/1000. The secondary antibody used was goat anti-rabbit IgG (H+L), HRP conjugate (#32460, Thermo Scientific) 1/1000.

2.1.6. RLU (Relative Light Unit) Measurement. A549/NF- κB -Luc cells treated for 24 h with betaine and/or C-PC were washed three times with PBS (Sigma-Aldrich), and DMEM without FBS was added along with human TNF α (Miltenyi Biotec) at final concentration of $25\text{ ng}\cdot\text{mL}^{-1}$ as an inductor of the NF- κB pathway. Following 9 h incubation, cells were washed once in PBS, trypsinised, mixed with complete medium, and centrifuged for 7 min at $1500\times g$ and the dried pellet was stored at -80°C until the day of analysis. The samples were thawed and washed with 500 μL PBS (5 min, $1500\times g$). The pellet was resuspended in 300 μL of Cell Culture Lysis buffer (Promega) with TritonX-100, vortexed

for 30 s, and incubated for 10 min at room temperature. Samples were centrifuged for 10 min at $3000\times g$, 60 μL of supernatant was put in the luminometer tubes on ice, and 100 μL mixture of lysis buffer with 10 mM ATP was added to each tube. Luciferase activity was measured using a luminometer (LUMAT LB 9507), normalized to total cell protein using a BCA protein assay kit (protein quantification kit—BC assays, Interchim, Montluçon Cedex, France) and expressed as relative light units (RLU) per mg protein.

2.1.7. Cell Cycle Analysis. A549 were synchronized in G_1 phase following FBS deprivation followed by thymidine inhibition of DNA synthesis. Cells were first incubated 24 h in DMEM without FBS, followed by 24 h in complete medium with 5 mM thymidine. At that point cells were synchronized in G_1 phase and were then incubated with C-PC and/or betaine. Following 24 h, cells were collected by trypsinisation, an equal amount of cells from each condition was centrifuged (5 min $3000\times g$), the pellet was gently resuspended in 300 μL of PBS, mixed with 700 μL of precooled 100% ethanol by gently inverting the tube, and stored at -20°C . On the day of analysis, the cells were pelleted (10 min, $5000\times g$), ethanol was removed, and 500 μL of PBS with 20 μL of propidium iodide ($50\text{ mg}\cdot\text{mL}^{-1}$, Becton Dickinson), 1 μL of RNase ($100\text{ mg}\cdot\text{mL}^{-1}$), and 0,01% of TritonX-100 were added. The suspension was transferred into FACS tubes (Falcon) and incubated for 30 min at room temperature protected from light. The samples were analysed by flow cytometry (Becton Dickinson FACSort).

2.2. In Vivo Study: Tumour Rat Model

2.2.1. Cell Culture. A549 cells (ATCC® CCL185™, human lung cancer) were maintained in culture by Cellvax Pharma, in DMEM environment, 10% Foetal Bovine Serum (FBS), 5 mM L-Glutamine, and 1% Penicillin/Streptomycin. A549 cells were grown to a confluence of 80%. For injection, A549 cells were suspended in Matrigel (50 : 50).

2.2.2. Study Design. A total of thirty-four pathogen-free male nude rats (Charles River, France), weighing $239.8 \pm 6\text{ g}$, aged 6-7 weeks, were used for this study. All animal work procedures were approved by the Institutional Animal Care and Use Committee of Maisons-Alfort (France).

Animals were acclimated for one week before initiation of the study. The daily light cycle extended from 7 a.m. to 7 p.m. and room temperature was maintained at $21.6 \pm 0.5^\circ\text{C}$. Throughout the study, rats were allowed to consume food and water ad libitum. Food contained 4% proteins and 3.9% fats, with the remainder being carbohydrates (A03, Society Safe diet, Augy, France). Rats were housed in individual cages.

Following acclimatization, 10^7 A549 cells were subcutaneously injected into the right flank of the rats.

Ten days following tumour implantation and once mean tumour volumes (MTV) reached 500 mm^3 , all animals were randomly assigned to nonintervention control group ($n = 8$; MTV: $568.8 \pm 98.9\text{ mm}^3$), betaine treatment group ($n = 9$; MTV: $568.5 \pm 133.8\text{ mm}^3$), C-PC treatment group ($n = 8$;

is involved in the synthesis of methionine, which serves as a major supplier of cellular cysteine via a transsulfuration pathway for the synthesis of reduced glutathione that protects the cell from reactive oxygen species [12]. It plays a central role in choline-mediated one-carbon metabolism, cell membrane structural integrity and signalling functions, and neurotransmitter synthesis. Betaine appears to be safe at a daily intake of 9–15 g [12]. Subacute and subchronic rat studies determined that betaine is nontoxic at all doses studied (0–5% of the diet) [13]. Betaine has been inversely associated with the risk of breast cancer [14], nasopharyngeal carcinoma [15], and colorectal cancer [16] and was also shown to decrease lung cancer risk [8]. Moreover, betaine supplementation seems to attenuate oxidative stress [17] and to have an anti-inflammatory effect through NF- κ B modulation and antiangiogenic effects [18]. Some articles have shown that betaine is able to inhibit the growth of cancer cells *in vitro* [19, 20] by regulating the expression of protooncogenes and tumour suppressors by stabilizing their methylation patterns [5]. Kim et al. [21] also recently reported that the effects of betaine are linked to the suppression of NF- κ B and Akt signal pathways *in vitro* and *in vivo*.

In vivo, a decrease in markers of oxidative stress (thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA)), conjugated dienes, carbonyl proteins, and nitrotyrosine was also noted by Basaran-Küçükgergin et al. [22] following 6 weeks of betaine supplementation (2.5% of chow) in rats. In liver cancer, betaine has a protective effect against the deleterious effects of chemotherapy using cisplatin by limiting the decline of antioxidant enzymes (glutathione disulfide reductase, glutathione peroxidase, catalase, and superoxide dismutase), by eliminating the increase in TBARS concentrations and by attenuating the proinflammatory and apoptotic mediators [23].

Besides betaine, phycocyanin (C-PC) is a protein from the phycobiliprotein family characterized by its intense blue colour and a structure consisting of a protein and nonprotein component known as phycocyanobilin. It is regarded as an ideal food and drug resource, due to its rich protein, lipid, vitamin, mineral, chlorophyll, β -carotene, and polysaccharide content. C-PC can be safely added to food, cosmetics, and medicine, with more and more studies confirming that C-PC has many biological activities such as antioxidation, antimitation, antitumour, and antiviral activities, immunity stimulation, hepatoprotective, antiplatelet, and neuroprotective properties and the scavenging of free radicals [24, 25]. Depending on the source, C-PC is classified into three groups: C-PC (from cyanobacteria), R-PC (from red algae), and R-PCII (from *Synechococcus* species) [26].

It appears that C-PC inhibits HeLa cell growth *in vitro*, in a dose-dependent manner by preventing DNA replication [27]. C-PC may induce the apoptosis of colon cancer cell SW480 and laryngeal cancer cell HEP-2 though the mechanism remains unknown [28, 29]. Li et al. [30] have reported that C-PC is an ideal cancer prevention drug as it may inhibit the growth of cancer cells *in vivo* and *in vitro*. Thus, C-PC may become a good substitute for highly toxic conventional chemotherapeutic anticancer drugs in the future [31].

To our knowledge, no studies have investigated the effect of betaine and C-PC on the growth, cell cycle distribution, or apoptosis of A549 cells, human pulmonary epithelial cell line derived from a lung adenocarcinoma, using both an *in vitro* and *in vivo* approach. Therefore, we investigated whether separate or combined treatment with betaine and C-PC would produce an inhibitory effect on the growth of A549 cell line, through a decrease of cell viability and whether this treatment would have an effect on the inflammatory NF- κ B pathway. Secondly, we wanted to evaluate whether the combination of C-PC and betaine would influence the p38 MAPK pathway or the cell cycle, as reported in previous studies separately for each compound [18, 32, 33]. Finally, we evaluated the effect of betaine and C-PC on the A549 tumour growth *in vivo* after oral supplementation of nude rats over a 4-week period (following the injection of A549 cells and tumour establishment).

2. Materials and Methods

2.1. In Vitro Study

2.1.1. Treatment Substances. C-PC from *S. platensis* (a gift from Greentech SA, Clermont-Ferrand) in powder form was stored at 4°C protected from light. Both betaine (B2754, Sigma-Aldrich) and C-PC stock solutions were prepared in a phosphate buffer saline (PBS) (Sigma-Aldrich), filtered through a 0.2 μ m pore filter and manipulated in a sterile environment. A 10% betaine stock solution (840 mM) was kept at 4°C, while a C-PC stock solution was stored at –20°C and protected from light.

2.1.2. Cell Culture. A549 (ECACC Cell Lines) and A549/NF- κ B-Luc (Panomics (ref RC0002)) cell line (A549 cell line with NF- κ B inducible promoter for luciferase) of human non-small-cell lung adenocarcinoma were cultured in Dulbecco's Modified Eagle Medium (Sigma-Aldrich), supplemented with 10% Foetal Calf Serum (FCS) (PAA Laboratories), 1% GlutaMAX (Gibco), and 1% Penicillin/Streptomycin (Sigma) at +37°C in 5% CO₂ atmosphere.

2.1.3. Sample Preparation. The cells (10⁴ cell/well in 96-well plate for cell viability assay, 6·10⁴ cell/well in 24-well plate for all other analysis, 2·10⁵ cell/T25 flask, or 6-well plate for cell cycle analysis and western blot, resp.) were seeded in 100, 400, 5000, and 3000 μ L medium, respectively, and cultured overnight. The medium was then removed and different concentrations and combinations of C-PC and betaine solutions were added to the cells and incubated for 24 h and 48 h at +37°C in 5% CO₂ atmosphere.

2.1.4. Cell Viability Assay. Following betaine and/or C-PC treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 20 μ L of 5 mg·mL⁻¹ solution in PBS) was added to each well containing 100 μ L of medium. The cells were then incubated for 4 h at +37°C [34]. MTT converted to formazan was solubilized with acidic isopropanol. The absorbance was measured at 562 nm with a Victor

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597.0 ± 136.4 mm³), and betaine + C-PC treatment group ($n = 8$; MTV: 703.4 ± 111.3 mm³).

2.2.3. Betaine Treatment. Betaine treatment was administered in drinking water and corresponded to 4% of the daily food intake (2.93 ± 0.3 g by kg of body weight). Betaine supplementation was adjusted every two days, according to the food and water intake of each rat. The daily food, water, and betaine intakes were evaluated for each rat.

2.2.4. C-PC Treatment. C-PC treatment was administered in drinking water and corresponded to 370.0 ± 0.03 mg by kg of body weight. C-PC supplementation was adjusted every two days, according to the food and water intake of each rat. The daily food, water, and C-PC intakes were evaluated for each rat.

2.2.5. C-PC and Betaine Treatment. Betaine and C-PC supplementation was administered in drinking water and corresponded to 4% of the daily food intake for betaine and to 370.0 ± 0.03 mg by kg of body weight for phycocyanin. The daily food, water, Betaine, and C-PC intakes were evaluated for each rat.

2.2.6. Evaluation of Tumour Growth. Tumour growth was measured (tumour length, width, and volume) twice a week by using an external calliper. Tumour volume was calculated by using the following formula: $V = \text{Length} \times \text{Width}^2/2$. The mean tumour volume (MTV) per group was estimated and given in the graph. The tumour growth data was recorded for each individually identified rat.

The observation period lasted 28 days. All experimental animals were euthanized if during that period the tumour volume reached 4000 mm³, as required by institutional guidelines.

At the 29th day, rats were removed from their cages, taken to an adjacent room, and within 30 seconds of removal from the cage were euthanized using anaesthetic (Ketamine: 75 mg·kg⁻¹ + Xylazine: 8 mg·kg⁻¹) and an aortic puncture. Immediately after euthanization, tumours were excised, weighed, and snap-frozen in liquid nitrogen and stored at -80°C.

3. Statistical Analysis

All statistical tests for the *in vivo* experiments were performed using the statistical package programme SPSS 19 (SPSS Inc., Chicago, IL). All values were expressed as the mean ± SE.

Nonparametric analyses (Kruskal-Wallis test and Mann-Whitney *U* test) were used in the statistical computations for tumour weight, tumour growth, food intakes, and body weight.

Statistical significance between groups for *in vitro* studies was assessed by the paired Student's *t*-test ($n = 3$) or repeated measures one-way ANOVA followed by Tukey's posttest (GraphPad Prism, San Diego, California, USA). Differences were considered statistically significant when $p < 0.05$.

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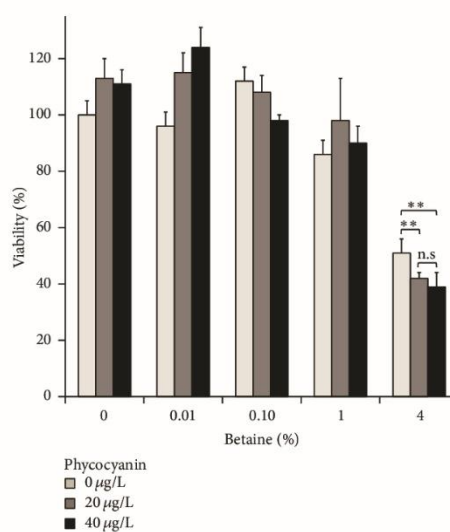


FIGURE 1: Decrease of cell viability following treatment with betaine and C-PC. Viability of A549 cells following treatment with 0%, 0.01%, 0.1%, 1%, and 4% betaine and 0 µg·L⁻¹, 20 µg·L⁻¹, or 40 µg·L⁻¹ of C-PC. Cell viability was measured using MTT assay after 48 h treatment. Mean ± standard deviation. ** $p < 0.005$; n.s stands for not statistically significant. Statistical significance between groups was assessed by the paired Student's *t*-test ($n = 3$).

4. Results

4.1. In Vitro Study

4.1.1. Viability of the A549 Cells. A549 cells were treated with betaine, C-PC, and a combination of both substances for 48 hours, followed by the viability evaluation through the MTT assay. After 48 h observing the sole betaine treatment, 4% betaine proved to be the most efficient, decreasing the viability of cells by 50%, so this was the one we chose for further application. Treatment with C-PC alone in tested concentrations did not yield a significant viability decrease in any dose or time dependency. On the contrary, combining 4% betaine with different C-PC concentrations decreased the viability by an additional 10–20% (Figure 1). The same effect was also observed for the C-PC concentrations not presented in the figure (data not shown).

4.1.2. Effect of C-PC and Betaine on the Inflammatory NF-κB Pathway. To study the effect of betaine and C-PC and their combination on the NF-κB pathway in the context of lung cancer, we used A549/NF-κB-LUC cells (Figure 2). In those cells, luciferase expression is driven by the inducible NF-κB promoter through activation of the NF-κB pathway. As a positive control and as an inducer of the NF-κB promoter activation, A549/NF-κB-LUC cells were treated with 25 ng·mL⁻¹ TNFα for 9 h.

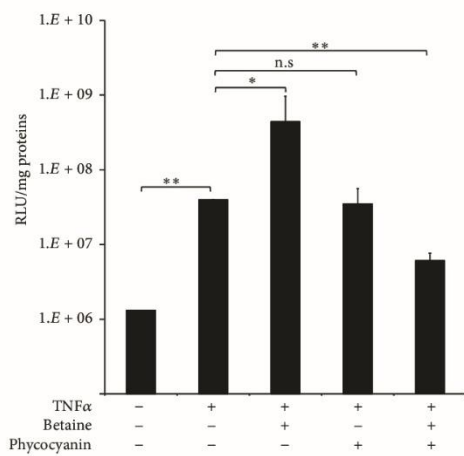


FIGURE 2: NF- κ B expression decrease with combined betaine/C-PC treatment. Measurement of luciferase activity in A549/NF- κ B-Luc cells after 24 h treatment with betaine and/or C-PC followed by 9 h treatment with TNF α . Mean \pm standard deviation. * $p < 0.05$, ** $p < 0.005$; n.s stands for not statistically significant. Statistical significance between groups was assessed by the paired Student's t -test ($n = 3$).

When cells were first treated for 24 h with C-PC and/or betaine and then further incubated for 9 h with TNF α , treatment with 4% betaine alone seemed to elevate the activation of NF- κ B. C-PC treatment alone did not have any significant effect while a significant decrease ($p < 0.05$) in activation was only observed when cells were treated with both 20 $\mu\text{g}\cdot\text{L}^{-1}$ C-PC and 4% betaine (Figure 2).

4.1.3. Effect of C-PC and Betaine on the p38 MAPK. Western blot was performed to evaluate the expression of p38 MAPK in A549 cells incubated with 4% betaine and/or 20 $\mu\text{g}\cdot\text{L}^{-1}$ C-PC. The expression of the p38 MAPK was evaluated by measuring the total p38 MAPK content after 24 h and 48 h and normalization to the β -actin. Values of the treatments are expressed as relative densities according to their respective controls.

Following 24 h incubation, there was no significant difference in any of the treatment groups (Figure 3(b)—condition B, grey bars). On the other hand, after 48 h, the increase in the expression can be seen in all conditions. While betaine alone and C-PC alone treatments show 1.5-fold increase, which is slight but insignificant, the combination of both gave an approximately 2.5-fold increase in the total p38 MAPK expression (Figure 3(b)—condition B + C-PC black bars). This increase was significant in relation to both 24 h B + C-PC treatment and the 48 h control MAPK expression.

4.1.4. Cell Cycle Analysis. To study the effect of C-PC and betaine and their combination on the cell cycle, A549 cells

were synchronized in G₀/G₁ cycle by serum deprivation followed by thymidine excess (Figure 4). Cell cycle distribution was evaluated following measurement of DNA content by propidium iodide and monitored by flow cytometry.

Asynchronous A549 showed standard distribution of cells in the different cell cycle phases, with G₀/G₁ as the most abundant (56.42%) and approximately equal distribution in S and G₂/M phase (21.77% and 20.92%, resp.) (Figure 4(a)—asynchronous). The synchronization protocol resulted in 91% of cells in G₀/G₁ phase, with almost complete blockage of mitosis (1.20%) and 7% of cells in S phase (Figure 4(a)—synchronized). G₀/G₁ synchronized cells were then either untreated or treated with betaine, C-PC, or a combination of both treatments for 18 h (Figure 4(b)).

After 18 h, untreated cells regained a cell cycle distribution similar to asynchronous cells (Figure 4(b)—untreated). 4% betaine treatment (Figure 4(b)—betaine) after 18 h induced an accumulation of cells in G₂/M phase (59.50% versus 17.54%) and in sub-G₀ (10.97% versus 0.72%) compared to untreated condition. The treatment with 80 $\mu\text{g}\cdot\text{L}^{-1}$ of C-PC lowered the percentage of cells in S phase (14.90% versus 21.64%) in favour of G₁ phase by 4% (Figure 4(b)—C-PC). The effect of combined treatment was close to that of betaine treatment alone even though C-PC seemed to enhance the accumulation in G₂/M by 5% as well as the decrease in S phase likely caused by betaine (Figure 4(b)—Betaine + C-PC). Furthermore, while the amount of cells in the sub-G₀ phase in nontreated cells and in C-PC treated cells remains negligible (<1%), betaine treatment counted 10.97% of sub-G₀ cells, either apoptotic or necrotic. When betaine was combined with C-PC, this number decreased to 6.71%. In conclusion, it would appear that 4% betaine induced cell cycle arrest in G₂/M phase and prevented further proliferation of cancer cells, as similarly observed in HeLa cells [35]. 80 $\mu\text{g}\cdot\text{L}^{-1}$ of C-PC only slightly influenced the cell cycle by increasing G₁ [36] and decreasing S phase cells. Interestingly, in combination with betaine, it improved the effect of betaine.

4.2. In Vivo Study. The body weights of rats were between 372.1 \pm 40.2 g and 350.8 \pm 42.8 g and were not significantly modified by treatments. Food intake was the same for all groups, suggesting that treatments have no toxic effects.

4.2.1. Effects of Betaine on Tumour Weight and Volume. Table 1 presents tumour volume and weight per group on the day of euthanization. Compared with the control group, tumour weight was significantly lower in betaine, C-PC, and betaine + C-PC treatment group. C-PC treatment induced a higher decrease in tumour weight as compared to betaine treatment. No further higher decrease was noted in the combination treatment group (betaine + C-PC).

Compared to the control group, a significant difference in tumour volume was noted (Figure 5) after the second week and continued to the end of the experiment.

Following 4 weeks of betaine treatment, tumour volume in the betaine group was 50% lower when compared to the control group, 88.2% lower for the C-PC group, and 86.8% lower for the betaine + C-PC group, respectively.

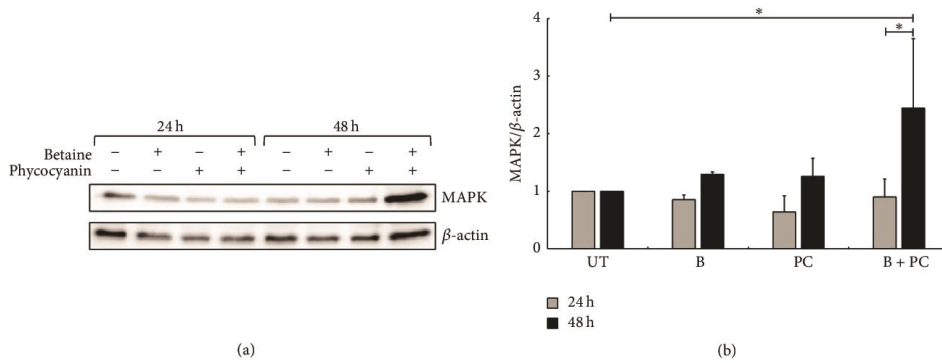


FIGURE 3: Activation of p38 MAPK pathway on A549 after betaine and/or phycocyanin treatment. Cells were treated with either 4% betaine, $20 \mu\text{g}\cdot\text{L}^{-1}$ of C-PC, or a combination of both molecules for 24 and 48 h. (a) Expression of p38 MAPK in different treatments was monitored by western blot. β -actin was used as a loading-control protein. (b) p38 MAPK along with β -actin band signals were quantified with the ImageJ software. After normalization with β -actin, p38 MAPK values were plotted on the graph as relative values compared to their respective controls. Bars are presented as the mean \pm standard deviation. * $p < 0.05$. Statistical significance between groups was assessed using repeated measures one-way ANOVA followed by Tukey's posttest. UT stands for untreated condition, B stands for betaine, and PC stands for C-PC.

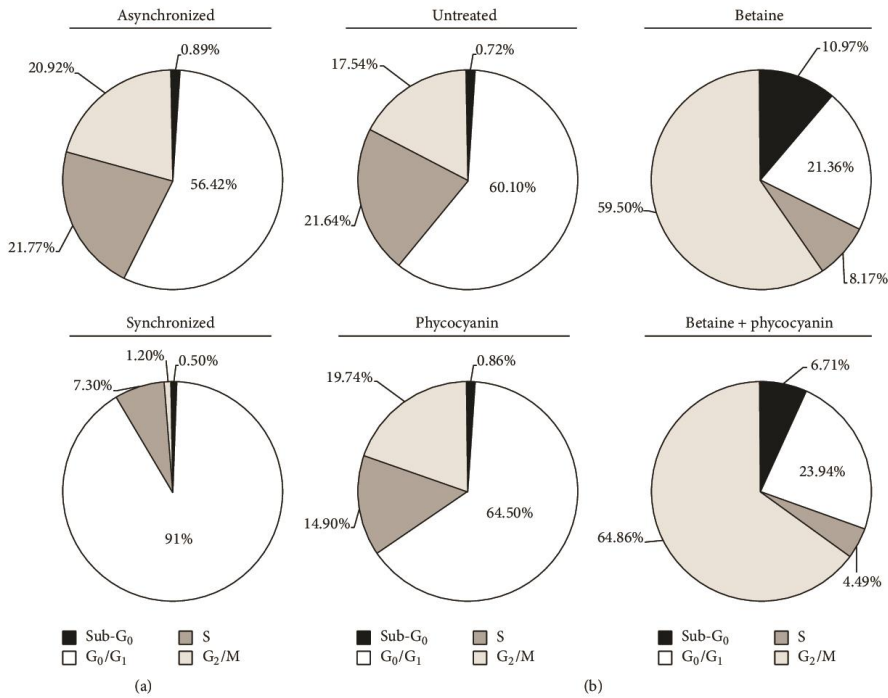


FIGURE 4: Betaine induced cell cycle arrest in G₂ phase. Cell cycle study of A549 cells treated with betaine and/or C-PC. (a) First, cells were synchronized in G₁ phase by serum deprivation followed by thymidine excess (synchronized). (b) Synchronized cells were then treated for 18 h with only medium (untreated), 4% betaine, $80 \mu\text{g}\cdot\text{L}^{-1}$ of C-PC, or a combination of the two treatments (betaine + C-PC). Cell cycles analysed by flow cytometry following propidium iodide staining are shown.

TABLE 1: Tumour volume in cubic millimetre (mm^3) and tumour weight in absolute values (g) and relative values (percentage of the body weight (% BW)) on the day of euthanization per group. Mean \pm standard deviation.

	Tumour volume (mm^3)	Tumour weight (g)	Tumour weight (% BW)
Control	4015 \pm 713	2.92 \pm 0.47	0.79 \pm 0.12
Betaine	1874 \pm 1154**	1.63 \pm 0.94*	0.48 \pm 0.28**
C-PC	473 \pm 162***SSS	0.21 \pm 0.06***SSS	0.06 \pm 0.02***SSS
C-PC + betaine	528 \pm 236***SSS	0.20 \pm 0.06***SSS	0.06 \pm 0.02***SSS

Compared to control group: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; compared to betaine group: SSS $p < 0.001$.

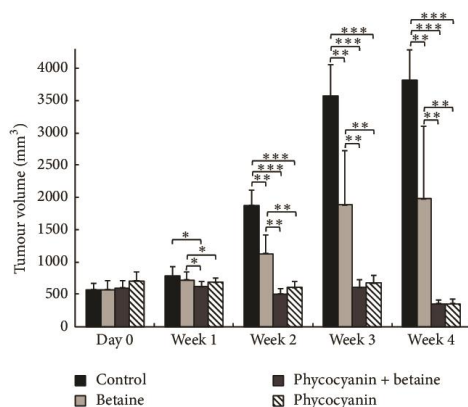


FIGURE 5: Evolution of mean of tumour volume per week per group, expressed in cubic millimetres (mm^3). Mean \pm standard deviation. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

5. Discussion

Cancer, also known as malignant tumour, is a disease that seriously damages health and lives. Recently, scientists have become interested in the potential antitumour effects of nutrients due to their safety and general acceptance. Some data have demonstrated that a large class of natural compounds, including pharmaconutrients, exhibit antitumour activities against selected cancer types [37, 38]. Among them is C-PC, a natural component of edible *S. Platensis* with no toxic side effects that is widely used as an excellent supplement to the human diet [39]. It could inhibit the growth of cancer cells both *in vivo* and *in vitro*. On the other hand, previous studies reported that betaine supplementation has an anti-inflammatory and antiangiogenic effect [18], as well as an anticancer action which may be explained through the regulation of the expression of protooncogenes and tumour suppressors by stabilizing their methylation patterns [40].

We showed that *in vitro* 4% betaine could effectively decrease the viability of A549 lung cancer cells, showing an even better effect if combined with C-PC. The effect observed with betaine correlated to a previous study reporting the decrease in viability following 2% and 4% betaine treatment of Hepal-6 cells (hepatocellular carcinoma) and HepG2 cells (human hepatoblastoma) [41]. Although there are previous

indications that C-PC alone can inhibit cancer cell growth in various concentrations and in a dose-dependent manner by inducing apoptosis [36, 42, 43], we did not observe this. One possible explanation could be the effectiveness of the product, which is highly dependent on the organism of origin, extraction and purification methods, and storage [44, 45].

Apoptosis can be induced through two distinct intracellular pathways mediated either by the death receptor or by the mitochondrial activation, the latter triggered as a response to environmental stress as well as to anticancer drugs [46]. Although weakly activated by growth factors, JNK and p38 MAPK respond strongly to stress signals [47, 48] and chemotherapeutic drugs, and their activation proved necessary for drug-induced apoptosis [49]. p38 MAPK is involved in the mitochondria-mediated apoptotic process and acts one step prior to mitochondrial dysfunction and caspase activation by influencing Bax translocation [50]. Its activation already proved important in the induction of apoptosis in nonsmall lung cancer cells [51]. Our western blot results support the MTT findings of the enhanced effect in combined B + C-PC treatment, showing that there is a greater impact on the expression of p38 MAPK when the two substances are used together, possibly activating apoptosis and reducing cell viability. Conversely, although betaine alone induces a significant viability decrease, there was no significant elevation of MAPK expression, leaving open the possibility of influencing other cellular pathways. Molecular mechanisms of C-PC-induced apoptosis have not been completely elucidated and possible mechanisms such as inhibiting COX-2 activity, reducing the expression of Bcl-2, enhancing the expression of CD59, and activating proteins from the cysteine protease family have been suggested [52]. Recently, Pan et al. [53] reported that C-PC may regulate the expressions of many kinds of proteins in SKOV-3 ovarian carcinoma cells to affect the process of apoptosis, oxidative stress, lipid, and ion transportation and finally inhibit the growth of tumour cells. Although the concentrations used may have been too low to show an effect after administration alone, it is not excluded that this was still enough to add to the effect of betaine by influencing one of the above mentioned pathways.

Disturbance of the cancer cell cycle is one of the therapeutic targets for development of new anticancer drugs [4]. Indeed, cell cycle regulation plays an important role in cell proliferation, differentiation, and apoptosis. In recent years, it has been reported that the dysfunction of cell cycle regulation appears to be closely connected to tumour onset and development. The G_2/M checkpoint allows the cell to repair DNA

damage before entering mitosis and is the most conspicuous target for many anticancer drugs that would cause cell death through the induction of apoptosis [54]. In this study, 4% betaine treatment induced cell cycle arrest in G₂/M phase, preventing a further proliferation of cancer cells, as similarly observed in HeLa cells [35], in correlation with the observed decrease of cell viability seen in the MTT assay. 11% of the cell distribution found in the sub-G₁ phase represents the population of cells in apoptosis or necrosis with fragmented nuclear DNA [55]. A successful anticancer compound should kill or incapacitate cancer cells without causing excessive damage to the normal surrounding cells. By inducing cell cycle arrest, the cells are more likely to enter apoptosis, a “clean” death without causing excessive inflammatory cell recruitment into the surrounding tissue through necrotic death, avoiding further damage to the tissue [56]. Incubation of cells with C-PC increased the cells in G₁ phase, as reported earlier [36, 57]. Wang et al. [58] have also demonstrated that C-PC could inhibit tumour cell proliferation and induce G₂/M phase arrest in HepG-2 cells. Ying et al. [54] reported that C-PC displayed a significant inhibition effect on the proliferation of human ovarian cancer cell SKOV-3 *in vitro*, which was a result of interactions among multiple pathways and signalling molecules. For these authors, interfering with a series of signalling pathways including proteoglycans in cancer, neurotrophin signalling pathway, VEGF signalling pathway, central carbon metabolism in cancer, and p53 pathway and many ending executive factors such as cyclin B1, cyclin-dependent kinase 2 (Cdc2) and Cdc25C, and C-PC influenced the proliferation and apoptosis of cancer cells. A combination with betaine increased the amount of both G₂/M phase cells and G₁ phase cells. In this case the sub-G₁ population was decreased by 4% compared to the betaine treatment alone. Since the sub-G₁ population contains both necrotic and apoptotic cells, increasing the amount of cells in the cycle arrest that can be guided more easily to apoptosis could bring greater benefit than having a higher amount of the sub-G₁ phase cells, where necrotic cells are potential triggers for inflammation, especially in an *in vivo* context. The molecular events controlling the G₁ phase of the cell cycle are determined by a series of phosphorylation events regulated by the expression of specific cyclins, cyclin-dependent kinases (CDK), and CDK inhibitors [59]. The activation of p38 MAPK was reported to lead to the G₂/M cell cycle arrest by suppressing CDC2 via the phosphatase CDC25 [60], which may be a connection between observed posttreatment increased expression of the p38 MAPK and the cell cycle arrest.

NF- κ B, although generally acknowledged as an antiapoptotic and proinflammatory mediator, along with TNF α has a complex role in cell signalling which is rather ambiguous [61]. It is already known that, after binding its receptor, TNF α induces several responses [62]. Apart from promoting the degradation of I κ B and releasing NF- κ B transcription factor, it activates two stress-activated MAPK pathways mediated by JNK and p38. Both can have a proapoptotic role [63] by activating caspase-3 of the intrinsic apoptotic pathway, which directly cleaves some of the proteins from the NF- κ B pathway, including NF- κ B [64]. In contrast to the expected

results, luciferase reporting system in A549/NF- κ B-Luc cells showed that 4% betaine enhanced TNF α activation of NF- κ B. However, it is important to keep in mind that this system reports only one side of the TNF α signalling pathway (only measuring the promoter activity of the NF- κ B gene and not the real content of the protein or mRNA in the cell) and that these same conditions in MTT assay showed a decrease in cell viability. This may lead to the assumption that, in this case, the “death” pathway of TNF α is more often activated or that NF- κ B here plays a proapoptotic role. In contrast to previous findings [65], C-PC applied alone did not show any inhibitory effect. The reasons may be the different, for example, C-PC purification methods, which influence the protein's properties as already mentioned, or may be due to different concentrations applied. Conversely, when C-PC was combined with 4% betaine it decreased activation of NF- κ B promoter (compared to the positive control). The future goal would be to further investigate at which instance and in what manner these two molecules, betaine and C-PC, interfere with the pathways of complex TNF α signalling.

To summarize, *in vitro*, betaine alone could inhibit the growth of tumour cells, while the combination of betaine and C-PC showed synergy. However, the effects of C-PC and betaine on lung cancer tumour *in vivo* need further study. In this research we used the NU/NU rat as an animal model to explore the effects of the two substances *in vivo*. Following tumour establishment, its weight and volume were monitored during the supplementation period in each group of rats. Compared with the control group, the tumour weight and volume were significantly reduced in the C-PC and betaine treatment group, respectively (Table 1 and Figure 5), and showed no further decrease in the combination group. In this case we observed no synergistic effect of the combined treatment but rather a strong inhibitory effect of C-PC alone. The beneficial effect of betaine could be due to several reasons. Its antioxidant properties are reported in many diseases and conditions, including oxidative stress induced by ethanol [17], restraint stress [12], and treatment of Parkinson's disease [66]. In mice with colon tumour, betaine specifically inhibited ROS generation and decreased GSSG concentration [21]. The other crucial role of betaine is as a methyl donor, modulating gene expression by targeting different elements of epigenetic mechanisms [67]. Esteller et al. [6] noticed that abnormal promoter DNA methylation was found in all stages of non-small-cell lung cancer, thus bringing betaine and its function into the spotlight. Furthermore, Lee [32] studied mouse hepatocyte H2.35 cells incubated with betaine and showed that betaine treatment leads to an upregulation of mitochondrial respiration and cytochrome c oxidase activity. He suggested that the antiproliferative effects of betaine on cancer cells might be due to enhanced mitochondrial function contributing to a reversal of the Warburg effect. The Akt signalling pathway is known to be associated with promotion of mitochondrial oxidative phosphorylation (OXPHOS) [32] and thus of mitochondrial respiration. Conversely, another study showed that inhibition of heme synthesis and mitochondrial respiration function can arrest the progression of lung cancer cells [68]. Even if the role of mitochondrial respiration is controversial, it seems, in any

case, to be one of the key factors in lung cancer progression. Considering the obtained *in vivo* effect of C-PC, we can find the support in earlier publications reporting its beneficial antitumour effects such as the scavenging of free radicals [26] or a concrete growth inhibition in different tumour cell lines, suggesting influence of various mechanisms and cellular pathways [27–30]. One possible explanation for why we did not observe the same inhibitory effect of C-PC *in vitro* could be the fact that the exact metabolism of phycocyanin is yet unknown. Bearing this in mind, it is possible that our *in vitro* concentration of C-PC was simply too low to produce an effect when administered alone.

Observing the obtained results *in vitro*, it is evident that there is some kind of interaction between C-PC and betaine that is still unknown. Since betaine transporters belong to the SLC36 group of proton coupled amino acid symporters that are pH sensitive [69], it is possible that C-PC as a cysteine/methionine rich protein [70] influences the uptake of betaine into the cells by adjusting the pH through the acid-base properties of its amino acid side-chains. Decrease of C-PC fluorescence inside the cells when combined with betaine (seen in the flow cytometry analysis—data not shown) may be explained by C-PC slightly changing the conformation after changing its amino acid charge by donating/accepting H⁺ ions. Also, it might be possible that C-PC interacts with betaine itself, since betaine is an amino acid-like compound, and that changing its charge influences its transport over the cell membrane or interactions inside the cell. However, further investigations are needed to confirm this hypothesis.

6. Conclusion

In summary, betaine and C-PC proved to be easily applicable and effective inhibitors of the A549 cell line growth. While in *in vitro* experiments the effect of C-PC was very slight or none, combining it with betaine seemed to enhance an already strong betaine effect. Thus, this combined treatment proved to be an effective inhibitor of the growth of A549 cells by inhibiting the progress of the cell cycle, reducing the cell viability, or enhancing the proapoptotic pathways. However, *in vivo*, both C-PC and betaine alone could successfully inhibit the growth of tumour in rats, and the tumour-suppressing effect was not more evident in the group receiving the combined treatment. Analysis of the methylation pattern following treatments, the oxidative status of cells and the organism, other cellular pathways involved, elucidation of the substance metabolism, and the nature of the interaction between betaine and C-PC, all these things, must still be answered to help us understand the underlying narrative.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential competing interests.

Acknowledgments

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Résumé de thèse en français

**Cancer bronchique non à petites cellules, immunité et microbiote :
Base d'étude de l'axe intestin-poumon-cancer du poumon chez
l'Homme**

Soutenue le 20 Décembre 2019 par

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1 Contexte et problématique

Selon l'Organisation Mondiale de la Santé (OMS), le cancer du poumon est la première cause de décès par cancer au monde, avec environ 1 761 000 décès enregistrés et 2 093 876 nouveaux cas en 2018 (International Agency for Research on Cancer, 2019). La classification se fait en deux groupes principaux : le cancer bronchique à petites cellules (CBPC) et le cancer bronchique non à petites cellules (CBNPC). Bien que le CBPC ait un moins bon pronostic et un taux de létalité très élevé, il ne représente qu'environ 5 % des cas de cancer du poumon.

Le CBNPC est donc plus fréquent et son pronostic dépend des mutations sous-jacentes, du type histologique et du degré d'agressivité (Molina et al., 2008). Dans le CBNPC, le principal outil de pronostic est la stadification des tumeurs d'après la classification TNM. Elle permet de classer les tumeurs en différents stades selon leur taille (T), les changements métastatiques sur les ganglions lymphatiques (N) et la métastase à distance (M) (Goldstraw et al., 2016). Les stades les plus avancés, en incluant notamment les changements métastatiques, sont associés à de moins bons résultats aux traitements et à une survie globale beaucoup plus courte (Planchard et al., 2019). Jusqu'alors, les mutations dites "d'évasion" étaient considérées comme la principale voie pour le cancer d'échapper à la réponse immunitaire de l'hôte. En effet, les cellules cancéreuses accumulent diverses mutations dues à leur taux élevé de renouvellement, à l'absence de suppresseurs de tumeurs et à des facteurs mutagènes extérieurs (p. ex. fumée, alcool) jusqu'à ce qu'elles deviennent non identifiables par le système immunitaire de l'hôte (Dunn et al., 2002).

Cependant, cette approche exclusivement intrinsèque commence à s'assouplir et la recherche sur le cancer adopte depuis peu une approche plus systémique. Plusieurs facteurs sont maintenant reconnus comme ayant une influence sur l'immuno-surveillance du cancer, tels que la nutrition, l'activité physique, le mode de vie (Molina et al., 2008), et plus récemment - le microbiote.

Le microbiote regroupe un consortium de bactéries, champignons, virus et protozoaires (Marsland et al., 2015), mais ce terme est le plus souvent utilisé dans la littérature pour désigner uniquement les bactéries (ce qui est également le cas dans ce manuscrit). Le microbiote du système gastro-intestinal est le plus étudié, mais il existe aussi des microbiotes résidant sur la peau et dans les autres cavités corporelles de l'hôte (tractus urogénital, zone oropharyngée,

etc.). Une reconnaissance accrue des fonctions qu'il exerce et son rôle crucial pour l'hôte font que le microbiote intestinal est souvent considéré comme un "organe oublié".

Avec une biomasse bactérienne de 2 kg, 1 à 10 fois plus de cellules que celles qui constituent notre corps et 100 fois plus de gènes que le génome humain, cet "organe" assure : l'homéostasie de l'hôte par la dégradation des nutriments, la synthèse de précurseurs et de vitamines, mais aussi la stimulation du système immunitaire (Bashiardes et al., 2017; Flint et al., 2012; García-Castillo et al., 2016; Kamada et al., 2013; Kau et al., 2011; Sender et al., 2016; Zeng et al., 2016). Le microbiote intestinal a été reconnu comme étant le facteur sous-jacent du développement du système immunitaire en bas âge (Gensollen et al., 2016; Thaïss et al., 2014), et sa dysbiose a été reconnue comme étant le facteur sous-jacent de plusieurs troubles immunitaires, comme l'asthme (Kalliomäki & Isolauri, 2003). On sait maintenant que les cellules bactériennes commensales stimulent constamment le système immunitaire, en maintenant un état de "veille" qui permettra de répondre plus efficacement à des antigènes étrangers, mais aussi en maintenant des mécanismes de "refroidissement" qui empêcheront une réaction excessive (Curotto de Lafaille et al., 2010; Mazmanian et al., 2005; Noverr & Huffnagle, 2004).

Cette caractéristique du microbiote commensal a été reconnue dans le traitement du cancer, ce qui a conduit à l'émergence de la théorie « intestin-lymphe » (Samuelson et al., 2015). Il suggère que les bactéries intestinales commensales pourraient stimuler l'immunité anti-tumorale non seulement localement, mais aussi de façon systémique. Ceci est possible grâce à la migration de bactéries, de leurs métabolites ou de cellules immunitaires stimulées vers l'emplacement distal via le système lymphatique. Au niveau du site distal, par exemple le lit de la tumeur, ces facteurs pourraient soit stimuler la réponse antitumorale (comme dans l'intestin), soit des cellules immunitaires déjà stimulées pourraient exercer directement leur effet antitumoral. La base de cette théorie repose sur plusieurs travaux menés sur des modèles animaux. Dans ces études, des animaux élevés dans des conditions exemptes de germes (GF) ou traités avec des antibiotiques n'ont pas répondu à la chimiothérapie classique avec du cyclophosphamide ou à la thérapie utilisant des inhibiteurs de point de contrôle immunitaire (Daillère et al., 2016; Routy, Le Chatelier, et al., 2018; Sivan et al., 2015; Viaud et al., 2013). En revanche, l'administration de souches bactériennes sélectionnées chez ces animaux a rétabli la réponse au traitement et a même eu un effet antitumoral si elles étaient administrées sans chimiothérapie (Sivan et al., 2015).

Ces études ont ouvert un vaste champ d'intérêt sur les oncobiotiques, des bactéries qui pourraient être utilisées comme médicaments anticancéreux et améliorer la surveillance immunitaire (Routy, Gopalakrishnan, et al., 2018).

Récemment, un nouvel axe "intestin-poumon" a été évoqué. De multiples observations suggèrent que la modification d'un régime alimentaire (associée à une modification du microbiote) ou l'exposition à certaines particules modifient la réponse immunitaire dans les poumons. La communication inverse est également possible. Sur cette base, la théorie de l'axe suggère une communication entre ces deux compartiments via la circulation lymphatique et sanguine. Afin d'aborder ces deux microbiotes très différents, de proposer une connexion basée sur la recherche à travers le système immunitaire, et d'aborder son application potentielle dans le cancer du poumon, nous avons écrit un **article de synthèse** intitulé "**Desired Turbulence ? Gut-Lung Axis, Immunity, and Lung Cancer**" (les auteurs dans l'ordre : **Rea Bingula, Marc Filaire, Nina Radosevic-Robin, Mathieu Bey, Jean-Yves Berthon, Annick Bernalier-Donadille, Marie-Paule Vasson, Edith Filaire**), publié dans *Journal of Oncology*, 17 septembre 2017.

Cet article a porté sur les connaissances actuelles concernant la composition du microbiote intestinal, rhino-oropharyngé et pulmonaire chez les sujets en bonne santé. Il a fait état de modifications du microbiote pulmonaire dans le cadre de la Bronchopneumopathie Chronique Obstructive (BPCO) et dans le cancer du poumon. Il a ensuite fourni une proposition détaillée d'interactions entre le système immunitaire de la muqueuse intestinale et le microbiote local, en tenant compte de l'influence des cellules bactériennes et de leurs métabolites. Cet article a également porté sur le concept bidirectionnel de l'axe intestin-poumon dans le cadre du cancer, fondé sur la « théorie de la lymphe » exposée précédemment, et incluant la théorie du cycle cancer-immunité.

L'intérêt accru pour les caractéristiques du microbiote et de son interaction avec l'hôte a conduit à la mise en place du Projet sur le Microbiome Humain (Human Microbiome Project - HMP). Ce projet vise à obtenir une caractérisation complète du microbiome humain grâce à la coopération de scientifiques du monde entier. Cependant, le microbiote pulmonaire n'a pas été initialement inclus dans ce projet (Proctor, 2011), ce qui a encore limité l'intérêt qu'on a pu lui accorder. Une autre difficulté rencontrée dans ce travail est liée à l'échantillonnage, en raison du caractère invasif des techniques employées. La plupart des études utilisent comme échantillon le liquide de lavage broncho-alvéolaire (LBA) obtenu par bronchoscopie, avec un risque de contamination par les voies respiratoires supérieures (Bassis et al., 2015; Beck et al.,

2012; Charlson et al., 2011). Jusqu'à présent, la plupart des études portaient sur le microbiote pulmonaire dans le cadre de le BPCO (Banerjee et al., 2004; Einarsson et al., 2016; Erb-Downward et al., 2011; Moghaddam, 2011; Sze et al., 2015), d'asthme (Gollwitzer & Marsland, 2014; Huang et al., 2011) et de la mucoviscidose (Fodor et al., 2012; Garg et al., 2017). En effet, dans le cadre de ces pathologies, une bronchoscopie de routine est souvent pratiquée ce qui simplifie le prélèvement d'échantillons de microbiote pulmonaire.

Ces dernières années ont été marquées par les premières études du microbiote pulmonaire dans le cas de cancer du poumon. Ces travaux ont mis en évidence une première interaction entre le microbiote pulmonaire et des facteurs environnementaux (Yu et al., 2016) et une seconde entre le microbiote pulmonaire et une inflammation accrue des voies respiratoires inférieures (Segal et al., 2016).

Même si certaines études évoquaient déjà l'influence "magique" du microbiote intestinal sur la réponse au cancer du poumon (Schuijt et al., 2016), la communauté scientifique a précisé qu'il fallait d'abord étudier l'effet du microbiote pulmonaire local avant de pouvoir évoquer le nouvel axe "intestin-poumon" (Dickson & Cox, 2017).

2 Objectifs et hypothèses

Avant d'élaborer une approche interventionnelle chez l'Homme basée sur des études antérieures chez l'animal, il est tout d'abord nécessaire d'étudier et de comprendre les facteurs coexistants dans la réponse de l'hôte au cancer du poumon. Pour ce projet de thèse, nous avons conçu un essai clinique incluant des patients atteints de CBNPC admissibles à une résection chirurgicale des tumeurs. Plusieurs objectifs ont été fixés :

- i) caractériser le microbiote de l'intestin, des poumons et des voies respiratoires supérieures chez ces patients**
- ii) évaluer l'homogénéité/hétérogénéité entre différents microbiotes dans le même sujet/groupe de patients**
- iii) évaluer l'impact de la composition du microbiote sur l'état immunitaire et inflammatoire du patient (évalué dans l'intestin, le sang et les poumons)**

Afin d'atteindre ces objectifs, nous avons inclus dans cette étude 17 patients et nous avons prélevé des échantillons de sang, de salive et de selles. Lors de l'admission et au cours des interventions chirurgicales, nous avons également prélevé quatre échantillons pulmonaires (Lavage Broncho Alvéolaire (LBA), tissu non cancéreux, tissus péri-tumoral et tumoral). Les échantillons salivaires, fécaux et pulmonaires (de 4 types) ont été utilisés pour l'extraction d'ADN et l'analyse du microbiote par qPCR et séquençage du gène de l'ARN 16S. Afin de limiter le risque de contamination du LBA par les voies aériennes supérieures, le lavage a été effectué directement sur le lobe excisé. Des échantillons de selles fraîches ont été utilisés pour le dénombrement microbiologique des groupes fonctionnels bactériens. Celles-ci ont également été congelées et utilisées ultérieurement pour doser les Acides Gras à Chaines Courtes (AGCC). L'état immunitaire a été évalué par la caractérisation de l'infiltration intra-tumorale en lymphocytes, par le phénotypage des lymphocytes Th et des sous-types de neutrophiles dans le LBA et le sang, par le dosage des cytokines dans le sang et par le dosage des marqueurs inflammatoires et antibactériens dans les selles. Chaque patient a également fourni une enquête nutritionnelle une semaine avant l'intervention chirurgicale afin d'aider à interpréter d'autres résultats (comme les quantités d'AGCC). D'autres renseignements cliniques et démographiques ont également été recueillis, comme le lieu de résidence, les antécédents de tabagisme ou la profession.

Notre première hypothèse a été que le microbiote pulmonaire diffère selon le lieu de prélèvement, même entre des échantillons proches histologiquement, comme les tissus péri-tumoraux et tumoraux. Les échantillons choisis pour l'analyse ont des fonctions physiologiques et/ou une architecture différente, mais aussi des interactions différentes avec le système immunitaire. Le LBA est représentatif de la population microbienne de la lumière bronchique, donc principalement une population bactérienne aux caractéristiques planctoniques, ou au contraire, associée aux biofilms ou mucus excrétés par les cellules alvéolaires. Le tissu non cancéreux représentera quant à lui, un échantillon avec une architecture pulmonaire normale faite de petites alvéoles et d'épithélium pulmonaire monocouche.

Selon le type histologique la tumeur peut varier, allant d'un tissu très dense comme dans le carcinome épidermoïde, à un tissu enrichi en mucus, comme dans certains types d'adénocarcinomes. L'augmentation de la production de mucus pourrait directement servir de nutriment aux bactéries mucinolytiques (Flynn et al., 2016) et ainsi favoriser la croissance de certains groupes bactériens. Enfin, l'échantillon de tissu péri-tumoral, sera considéré comme un échantillon bien distinct car il possède des caractéristiques histologiques différentes. Même si, de par son architecture, le tissu péri-tumoral ressemble à un tissu non cancéreux, sa proximité avec la tumeur le rend exposé à diverses substances excrétées par les cellules tumorales et à une invasion possible des cellules tumorales elles-mêmes (Dou et al., 2018).

Un autre aspect important est le rôle du tissu péri-tumoral, c'est-à-dire du microenvironnement tumoral (MET), sur la suppression ou la stimulation directe de la tumeur de diverses manières: par la modification de la composition de la matrice du MET, par la production de facteurs de croissance, etc. (Valkenburg et al., 2018). Ces deux événements pourraient directement (avec des changements dans l'architecture des tissus) ou indirectement (avec une réponse immunitaire contre les cellules tumorales) influencer le microbiote local.

Nous avons ensuite émis l'hypothèse qu'il y aurait plusieurs facteurs potentiellement discriminants pour le système immunitaire et le microbiote dans les six échantillons prélevés (salive, selles et les quatre échantillons pulmonaires).

Le premier facteur est le type histologique de tumeur ; il a déjà été démontré que le type histologique modifie le microbiote salivaire des patients atteints de cancer du poumon (Yan et al., 2015), mais également l'alpha diversité dans les tissus tumoraux (Yu et al., 2016). Les différents types histologiques vont avoir un impact direct sur le microbiote tel qu'expliqué dans le paragraphe précédent. Ils sont aussi caractérisés par une immunogénicité, une nature

immunosuppressive et un fond génétique différents (Busch et al., 2016), ce qui signifie qu'ils ne stimuleront pas de la même manière le système immunitaire. Par conséquent, l'afflux et le profil des cellules immunitaires vers le lit tumoral varient, de même que l'interaction avec le microbiote local. La nature de la tumeur et la situation immunitaire locale pourrait se refléter sur les marqueurs systémiques, et donc sur l'état immunitaire intestinal. Par conséquent, le microbiote intestinal peut aussi varier selon le type de tumeur. Cependant, dans ce type d'étude, il n'a pas été possible de déterminer si ce changement est la conséquence d'une maladie, d'un facteur concomitant ou le résultat d'un changement dans l'équilibre immunitaire.

Le deuxième facteur important est le statut tabagique du patient. La fumée de cigarette présente plusieurs effets indésirables sur le système respiratoire, comme l'inhalation d'air chaud et la précipitation de particules potentiellement cancérigènes. Ceci endommage les cellules épithéliales et altèrent la clairance des voies respiratoires, induisant une inflammation locale et systémique (Çolak et al., 2019). En résulte une modification des conditions locales de croissance pour le microbiote et une perturbation de l'homéostasie immunitaire. Les voies respiratoires supérieures sont affectées par la fumée, et des changements de microbiote liés au tabagisme ont déjà été rapportés (Wu et al., 2016). Fait intéressant, il n'a pas été observé de changements dans les voies respiratoires inférieures (Segal et al., 2013). Ainsi, il est possible que les microbiotes des différents sites pulmonaires ne soient pas sensibles de la même manière aux effets du tabagisme. Cette hypothèse sera vérifiée dans ce travail. De plus, il a été observé que le tabagisme modifiait le microbiote intestinal, tant chez les personnes en bonne santé (Stewart et al., 2018) que chez les patients atteints de cancer du poumon (Zhang et al., 2018; Zhuang et al., 2019), ce qui montre clairement l'effet du tabagisme au niveau systémique.

Un autre facteur important est l'emplacement de la tumeur. Les poumons sont organisés en cinq lobes, deux du côté gauche (supérieur et inférieur) et trois du côté droit (supérieur, moyen et inférieur). Il est intéressant de noter que des taux de survie globale plus faibles et un pronostic plus défavorable ont été associés à des tumeurs dans les lobes inférieurs, et particulièrement dans le lobe inférieur gauche (Kudo et al., 2012). Le modèle insulaire adapté à la biogéographie pulmonaire des sujets sains suggère déjà que le microbiote pulmonaire est différent entre les lobes (Dickson et al., 2015). Ce modèle décrit la bronche principale comme étant la source des communautés microbiennes pulmonaires, dont la richesse diminue proportionnellement à l'éloignement de ce site. Par conséquent, les lobes supérieurs devraient être plus riches en microorganismes que les lobes inférieurs, mais les lobes controlatéraux devraient avoir des caractéristiques similaires. Cependant, l'étude qui compare le LBA controlatéral (c'est-à-dire du

lobe gauche et du lobe droit), l'un cancéreux et l'autre non, montre que ce n'est pas le cas pour le cancer du poumon (Liu et al., 2018). Il est évident que la présence de tumeurs modifie la composition microbienne et que d'autres études incluant des patients atteints de cancer du poumon seront nécessaires.

Nous nous sommes ensuite concentrés sur un autre facteur important ayant un rôle pronostique crucial dans le traitement du CBNPC : la stadification de la tumeur. Plus le stade est élevé, plus le pronostic global pour le patient est mauvais. Cependant, le stade est déterminé par la combinaison des trois éléments dans la classification TNM : la taille de la tumeur T, la présence de métastases au niveau des ganglions lymphatiques médiastinaux et ipsilatéraux N, et la présence de métastases distantes M (Goldstraw et al., 2016).

Un même stade peut donc parfois représenter des tumeurs véritablement différentes. Par exemple, une très grosse tumeur sans ganglions lymphatiques métastatiques pourrait appartenir au même stade qu'une petite tumeur mais avec des ganglions métastatiques. Pour les besoins de l'étude du microbiote, nous avons décidé d'observer séparément ces éléments et de définir l'hypothèse que chacun d'entre eux pourrait influencer individuellement nos communautés microbiennes.

Étant donné que les patients présentant des métastases à distance ne sont pas inclus dans l'étude en raison de la différence dans la procédure de traitement (non opérable), nous avons considéré deux autres éléments : la taille de la tumeur et la présence de ganglions lymphatiques métastatiques. Selon le diamètre, la tumeur peut être plus ou moins oxygénée en raison d'une vascularisation souvent très fuyante et insuffisante pour les tumeurs de plus grandes tailles, avec par conséquent un degré de nécrose interne différent (Wouters et al., 2003). Ceci favoriserait la colonisation de bactéries anaérobies qui pourraient soit défavorablement diminuer le pH par production de lactate, soit, favorablement augmenter l'immunogénicité tumorale (Baban et al., 2010; Damgaci et al., 2018; Van Dessel et al., 2015). Des tumeurs plus grosses pourraient également bloquer les voies bronchiques et ainsi créer des poches micro-aérobies ou anaérobies. Ceci favoriserait les changements dans la composition microbienne ou la colonisation par des agents pathogènes.

La présence de ganglions lymphatiques métastatiques est, en revanche, le signe d'une plus grande agressivité tumorale et d'une fuite vasculaire permettant la dissémination des cellules tumorales aux ganglions lymphatiques proximaux quelle que soit la taille de la tumeur. L'existence de ces ganglions lymphatiques métastatiques dénote le caractère invasif de la

tumeur et signale également un microenvironnement "permettant" la propagation des cellules tumorales. Selon le Dr Bissell (Bissell & Hines, 2011), l'environnement tumoral joue un rôle énorme dans le développement et le devenir de la tumeur. Dans le cadre de notre étude, certaines bactéries de l'environnement tumoral pourraient par exemple occuper les sites d'adhésion de différentes intégrines cellulaires (Garrett, 2015; Khan et al., 2012). L'occupation de ces sites inhiberait la diapédèse métastatique des cellules tumorales. La présence de ganglions lymphatiques métastatiques étant un marqueur important de pronostic négatif pour la survie, et ce quelle que soit la taille de la tumeur (Liu et al., 2017). Elle a été priorisée dans notre analyse.

On sait que les patients atteints de cancer, en particulier lors de stade avancé de la maladie, signalent souvent une perte de poids sans changements particuliers dans leur alimentation. Ce phénomène appelé cachexie s'explique, d'une part par la présence de cellules cancéreuses voraces qui peuvent capter la majeure partie de l'énergie de l'hôte obtenue par digestion, et d'autre part par différentes cytokines, qui produites par l'hôte en réponse au cancer peuvent modifier son métabolisme (Barton, 2001). Des changements au niveau du microbiote intestinal liés à la présence du cancer, ainsi que l'utilisation de probiotiques dans la lutte contre la cachexie ont été envisagés, mais il reste nécessaire de poursuivre les recherches dans ces domaines (Bindels & Thissen, 2016). Par conséquent, notre hypothèse concernant l'effet du stade tumoral sur le microbiote pulmonaire s'est également étendue au microbiote intestinal (en tenant compte des informations sur la stabilité pondérale du patient et de leur étude nutritionnelle pour une approche personnalisée).

Enfin, la théorie de l'axe intestin-poumon implique que certains éléments d'origine intestinale, comme les bactéries ou leurs métabolites, amorceraient les cellules immunitaires situées dans les ganglions mésentériques. Ces cellules immunitaires seraient ensuite recrutées par des sites éloignés où elles pourraient agir avec plus de performance (Bingula et al., 2017). Dans notre cas, cette performance accrue pourrait être caractérisée par une meilleure infiltration des cellules immunitaires dans les tumeurs pulmonaires. Cependant, il est important de garder à l'esprit qu'il existe trois types généraux de tumeurs : les tumeurs immuno-inflammées (angl. « immune-inflamed »), les tumeurs immuno-exclues (« immune-excluded ») et les tumeurs immuno-désertes (« immune-desert ») (Chen & Mellman, 2017). Sommairement, les tumeurs immuno-inflammées sont bien infiltrées et immunogènes et les tumeurs immuno-désertes sont mal infiltrées et peu immunogènes. Les tumeurs immuno-exclues sont quant-à-elles souvent immunogènes mais avec certains éléments intrinsèques, comme l'expression du PD-1 (Death Domain Protein-1), qui inhibent l'infiltration et l'activité des cellules immunitaires.

Théoriquement, il serait possible de passer d'un type d'immunité tumorale à l'autre. Par exemple une tumeur immuno-inflammée qui, après intervention du système immunitaire, ne conserve que des cellules faiblement immunogènes pourrait devenir immuno-déserte. L'inverse est également possible : un meilleur amorçage des cellules immunitaires pourrait transformer une tumeur immuno-déserte en une tumeur immuno-inflammée grâce à une réactivité croisée entre les cellules tumorales et les épitopes bactériens. Par conséquent, notre hypothèse a reposé sur le fait que le microbiote intestinal, mais aussi le microbiote pulmonaire, varierait selon le type immunitaire tumoral.

Les hypothèses avancées seront développées plus en détail au fur et à mesure de l'avancement des analyses. Les hypothèses exposées comprennent des facteurs ayant une influence à la fois locale et systémique sur l'hôte, et donc sur son microbiote et son système immunitaire. C'était la raison sous-jacente pour laquelle il fallait aborder les patients sous différents angles (microbiote de trois sites, marqueurs immunitaires et inflammatoires locaux et systémiques par différentes techniques, métabolites bactériens, nutrition et données démographiques). Les objectifs et les hypothèses que nous allons vérifier seront discutés dans les chapitres "Résultats" et "Discussion générale".

3 Résultats principaux

Study Protocol

Characterisation of Gut, Lung and Upper Airways Microbiota in Patients with Non-Small Cell Lung Carcinoma: Study Protocol for Case-Control Observational Trial

Rea Bingula, MS, Marc Filaire, Prof, MD, Nina Radosevic-Robin, MD, Jean-Yves Berthon, PhD, Annick Bernalier-Donadille, PhD, Marie-Paule Vasson, Prof, Emilie Thivat, PhD, Fabrice Kwiatkowski, MS, Edith Filaire, Prof

➤ Publié dans *Medicine* le 14 Décembre 2018

La majorité des études explorant le microbiote pulmonaire par séquençage à haut débit utilisent, pour l'isolement du microbiote, des kits commerciaux qui ne sont pas spécialement adaptés aux échantillons pulmonaires (exemple : MoBio PowerSoil ou QIAamp Stool Minikit de Qiagen). Étant donné que les échantillons pulmonaires diffèrent significativement en qualité (tissu très fibreux) et en niveau de populations bactériennes par rapport aux fèces ou au sol, ces kits ne sont pas les mieux adaptés et leur rendement final est insuffisant (utilisation de colonnes de filtration, etc.). C'est pourquoi nous avons décidé de mettre au point un protocole de "isolement du microbiote pulmonaire", basé sur le protocole du Human Microbiome Project pour l'isolement du microbiote intestinal ; protocole dont la performance a précédemment été comparée à d'autres kits disponibles sur le marché (Costea et al., 2017; Mcinnes, 2010). La version finale optimisée du protocole, comprenant la conception de l'étude, et le matériel et les méthodes utilisés ont été publiés dans l'article suivant : "Caractérisation du microbiote de l'intestin, des poumons et des voies respiratoires supérieures chez les patients atteints de cancer du poumon non à petites cellules" publié dans *Medicine* le 14 décembre 2018. Seule la partie du projet concernant les patients admissibles à la chirurgie sans chimiothérapie sera présentée dans ce rapport de thèse, et son aperçu synthétique est présenté en Figure 1.

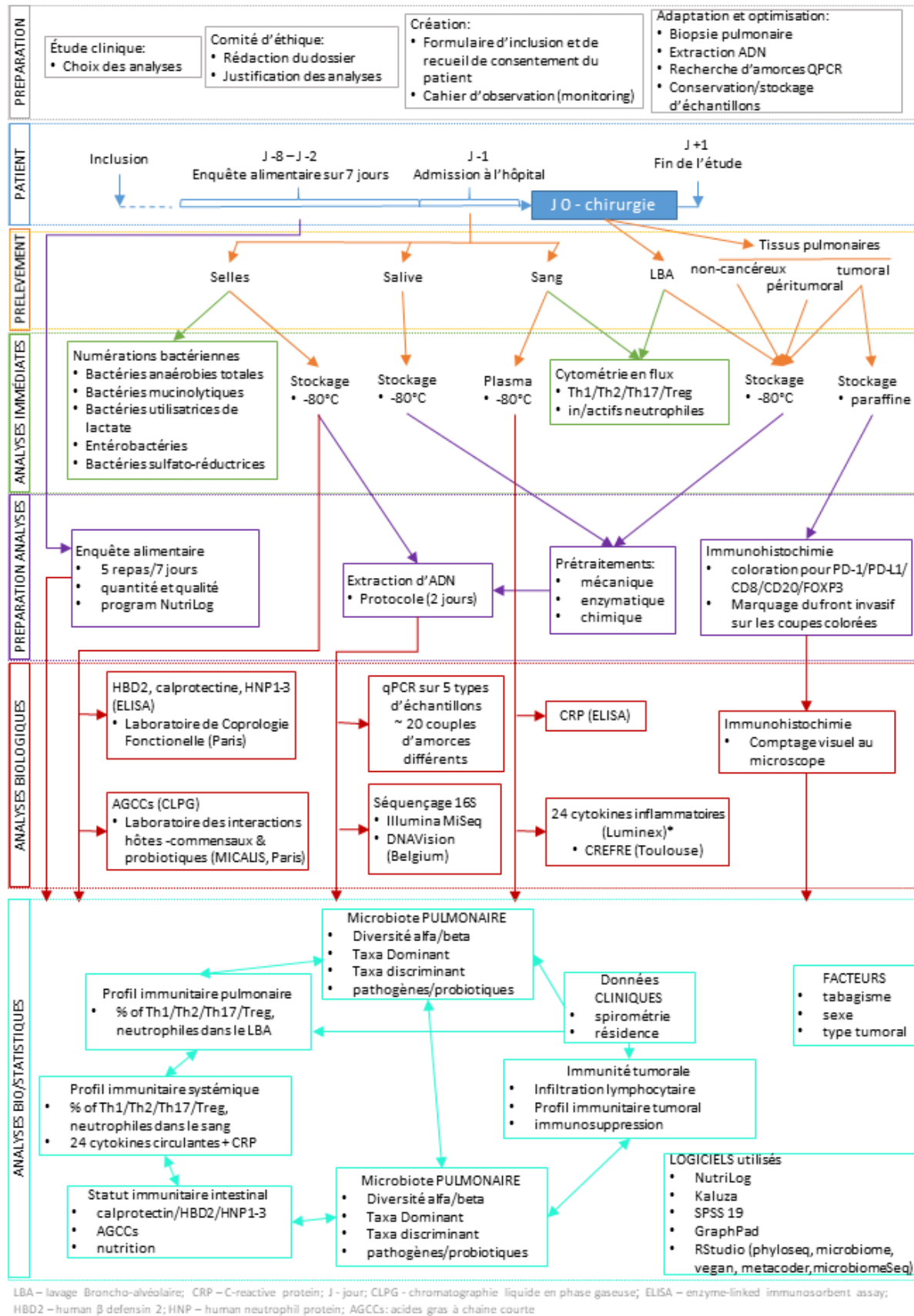


Figure 1 Aperçu synthétique d'étude

Article 1

Research Article

Characterisation of microbiota in saliva, bronchoalveolar lavage fluid, non-malignant, peritumoural and tumour tissue in non-small cell lung cancer patients: cross-sectional clinical trial

Rea Bingula, Edith Filaire, Ioana Molnar, Eve Delmas, Jean-Yves Berthon, Marie-Paule Vasson, Annick Bernalier-Donadille, Marc Filaire

➤ Soumis dans *BMC Respiratory Research* le 05 Décembre 2019.

Introduction

Même si des études portant sur le microbiote du poumon cancéreux ont commencé à émerger il y a plusieurs années, les caractéristiques du microbiote pulmonaire provenant de différents types d'échantillons ne sont pas encore bien documentées. Le principal problème de la recherche sur le microbiote pulmonaire provient de différentes méthodes d'échantillonnage qui sont à l'origine de l'hétérogénéité des échantillons cibles d'une étude à l'autre. Ainsi la majorité des études actuelles portant sur le microbiote du poumon ont été réalisées sur le LBA, les tissus, la salive et les expectorations à fréquence variable. Bien qu'il ait déjà été suggéré que le microbiote pulmonaire provenant du LBA et des tissus pulmonaires abrite des communautés différentes (Sze et al., 2012), aucune étude n'a encore été réalisée sur ce sujet. De plus, l'analyse du microbiote dans le cadre de cancer du poumon implique des tissus ayant des rôles, des interactions et une immunité distincte, comme les tissus péritumoraux et tumoraux. Par conséquent, l'objectif de cette étude était d'une part, de fournir une caractérisation détaillée du microbiote pulmonaire oral et d'autre part, d'obtenir de nouvelles données innovatrices dans ce domaine. Puisque les tumeurs pulmonaires des lobes inférieurs (LL) ont été associées à une diminution de la survie, les caractéristiques du microbiote des lobes supérieurs (UL) et inférieurs ont également été examinées.

Matériels et méthodes

À l'aide de la technologie de séquençage du gène 16S de l'ARNr, nous avons analysé le microbiote dans la salive, le LBA (obtenu directement sur le lobe excisé), les tissus non cancéreux, péritumoraux et tumoraux de 18 patients CBNPC admissibles à un traitement chirurgical. Nous avons fourni : une taxonomie détaillée, la diversité et les principaux groupes

bactériens pour chaque microbiote, avec analyse de l'abondance différentielle à tous les niveaux taxonomiques entre les échantillons et en fonction de la localisation des prélèvements dans les poumons (modèle linéaire général binomial « zero-inflated » avec correction Benjamini-Hochberg).

Résultats, discussion et conclusion

Nous démontrons que le LBA représente un microbiote unique en comparaison avec les autres échantillons pulmonaires. Ce résultat est basé sur l'analyse de l'abondance différentielle et sur la Bêta diversité. Simultanément, nous confirmons que les échantillons de LBA forment un groupe distinct avec les autres échantillons de tissus pulmonaires lorsqu'ils sont placés dans la perspective du microbiote salivaire. La position du lobe est un facteur important influençant la composition du microbiote (hypothèse du modèle insulaire adapté à la biogéographie pulmonaire) et en même temps, les tumeurs du lobe inférieur ont été associées à un pronostic moins favorable chez les CBNPC. Sur cette base, nous avons fait le choix d'examiner l'influence de la position du lobe supérieur ou inférieur sur le microbiote analysé. Nous montrons ainsi que tous les microbiotes analysés, à l'exception du microbiote tumoral, ont une abondance significativement différente, en particulier dans les phyla *Bacteroidetes* et *Actinobacteria*. De plus, nous montrons que le microbiote du tissu péri-tumoral est le plus susceptible au changement selon son emplacement, avec une ressemblance significativement accrue au microbiote du LBA lorsqu'il se trouve dans les lobes supérieurs. Puisque la tumeur est connue pour son potentiel à modifier directement son microenvironnement, nous faisons l'hypothèse que ce changement observé dans le microbiote des tissus péri-tumoraux reflète en fait les changements du microenvironnement (sites adhésifs, réarrangement de la matrice extracellulaire, stimulation immunitaire). Enfin, nous montrons que le phylum des *Firmicutes*, précédemment signalé comme étant un phylum abondant dans les tumeurs pulmonaires évolutives, est également élevé en abondance dans tous les tissus pulmonaires des lobes inférieurs par rapport aux lobes supérieurs. Il pourrait s'agir d'un potentiel indicateur microbien d'une agressivité accrue des tumeurs du lobe inférieur, mais il faudra toutefois confirmer cette hypothèse en incluant notamment davantage les caractéristiques du système immunitaire.

Article 2

Research Article

Inverse abundance pattern of tumour and extratumoural lung microbiota between non-small cell lung cancer patients with or without metastatic lymph nodes: a cross-sectional clinical study

Rea Bingula, Edith Filaire, Jérémie Talvas, Jean-Yves Berthon, Marie-Paule Vasson, Annick Bernalier-Donadille, Nina Radosevic-Robin, Marc Filaire

➤ Soumis dans *BMC Microbiome* le 25 Octobre 2019

Introduction

La présence de ganglions lymphatiques métastatiques (LN) est l'un des marqueurs de pronostic négatif les plus importants dans le CBNPC (Goldstraw et al., 2016). Il est caractérisé par un temps de survie beaucoup plus court et par un taux de récurrence accru. Nous avons cherché à savoir si cette caractéristique importante pour le devenir du patient est associée de façon significative à la composition locale du microbiote pulmonaire. Nous nous sommes basés sur l'analyse du microbiote pulmonaire de quatre échantillons différents (LBA, tissus non cancéreux, péri-tumoraux et tumoraux). De plus, nous avons examiné si ces deux paramètres (présence ou absence de métastases ganglionnaires) influencent l'immunité locale en étudiant la composition de l'infiltrat immunitaire tumoral et les phénotypes des lymphocytes Th dans le LBA. Enfin, nous avons cherché à savoir si cette réponse immunitaire est associée à différents microbiotes pulmonaires.

Matériels et méthodes

Le microbiote pulmonaire a été analysé en LBA (lavage direct du lobe excisé) et dans trois tissus (tissus non cancéreux, péri-tumoraux et tumoraux) par séquençage du gène de l'ARN ribosomal 16S, suivi par l'analyse biostatistique en RStudio. Le statut immunitaire a été évalué en dénombrant les lymphocytes infiltrant les tumeurs (marqueurs de l'expression des CD8, CD20, FOXP3, PD-1 et PD-L1) et en déterminant les profils des lymphocytes T-Auxiliaires et les neutrophiles dans le LBA. Dans la population lymphocytaire, la sous-population de lymphocytes T-Auxiliaires CD3⁺CD4⁺ a été caractérisée comme suit : Th1 avec marqueur CXCR3⁺, Th2 avec marqueur CRTH2⁺, Th17 avec marqueur CCR6⁺ et Treg avec marqueurs CD25⁺CD127⁻. Dans la population de granulocytes, les sous-populations de neutrophiles ont

été caractérisées comme suit : neutrophiles circulants avec marqueurs CD15⁺CD62L⁺CD11b⁻, forme transitoire avec marqueurs CD15⁺CD62L⁺CD11b⁺, et neutrophiles activés avec marqueurs CD15⁺CD62L⁻CD11b⁺. Toutes les analyses statistiques ont été réalisées avec RStudio.

Résultats, discussion et conclusion

Nous montrons qu'en présence de ganglions lymphatiques métastatiques, tous les échantillons de microbiote pulmonaire présentent une abondance accrue du phylum des *Firmicutes*, tandis que les mesures de diversité montrent des valeurs plus faibles pour les deux microbiotes des tissus extratumoraux. Nous avons identifié 31 genres bactériens présentant des schémas inverses d'abondance entre les tissus tumoraux, extratumoraux, et entre les patients avec et sans ganglions lymphatiques métastatiques. De plus, nous montrons que cette inversion est étroitement liée au profil respiratoire bactérien. Plus précisément, les genres anaérobies identifiés sont significativement plus abondants dans les tumeurs à ganglions lymphatiques métastatiques, alors que les bactéries aérobies sont plus abondantes dans les tissus extratumoraux. Cependant, nous observons exactement le phénomène inverse pour ces mêmes genres bactériens chez les patients ne présentant pas de ganglions lymphatiques métastatiques. Nous avançons donc l'hypothèse que les bactéries "migrent" entre les tissus vers des conditions de croissance plus favorables, car l'environnement tumoral change fortement en fonction de sa progression (hypoxie accrue, nécrose, production de lactate, pH faible). De plus, nous identifions plusieurs biomarqueurs potentiels dans chaque tissu qui pourraient faciliter ou améliorer la détection des métastases des ganglions lymphatiques sans imposer de biopsie. Enfin, nous montrons que le microbiote du LBA ne change pas de façon significative en fonction du stade tumoral, mais qu'il est le seul à présenter une association significative avec les marqueurs immunitaires protumoraux ou antitumoraux dans le LBA et dans la tumeur.

4 Discussion, conclusion et perspectives

4.1 Discussion

En raison des multiples axes de recherche possibles dans ce projet, nous avons d'abord décidé de nous concentrer sur la région pulmonaire, moins explorée que la région intestinale. C'est pourquoi nous nous sommes focalisés dans un premier temps sur la caractérisation des différents microbiotes pulmonaires (tumeur, tissu péri-tumoral et non cancéreux, et LBA), leur relation avec le microbiote salivaire et l'impact de la localisation du lobe tumoral (Article 1). Dans un second temps, afin d'envisager une application clinique directe de notre recherche, nous avons donc cherché à déterminer s'il existe une interaction entre : la présence de ganglions lymphatiques métastatiques (l'un des principaux facteurs pronostiques du CBNPC), différents microbiotes pulmonaires et l'immunité locale (Article 2). D'autres axes de recherche seront abordés dans « Conclusion et Perspectives ».

- ❖ Le LBA représente un microbiote pulmonaire unique. Il n'est que sensiblement plus semblable à la salive que les tissus pulmonaires.

Depuis le début des recherches portant sur l'étude du microbiote pulmonaire, plusieurs inconvénients sont apparus. L'un des plus importants reste le choix de l'échantillon pulmonaire qui représenterait correctement les communautés pulmonaires, mais qui ne serait pas trop invasif pour le patient. Par conséquent, le LBA était et demeure le type d'échantillon le plus fréquemment utilisé dans les études portant sur le microbiote pulmonaire en général et dans les cas de cancer du poumon (Laroumagne et al., 2011; Lee et al., 2016; Liu et al., 2018; Tsay et al., 2018; Wang et al., 2019). Cependant, il a déjà été suggéré que le microbiote pulmonaire présent dans le LBA et dans les tissus pulmonaires ne partagent pas les mêmes caractéristiques (Sze et al., 2012), en raison de la physiologie différente des échantillons.

Nous sommes les premiers à avoir montré que le LBA abrite un microbiote distinct de celui du tissu pulmonaire, en incluant dans nos analyses jusqu'à trois échantillons de tissus différents (tissu non cancéreux, péri-tumoral et tumoral) (Article 1). Le LBA représente un échantillon unique, en se basant à la fois sur la Bêta diversité et sur l'analyse de l'abondance différentielle. Dans le LBA, les *Firmicutes* représentent le phylum le plus abondant. En revanche, les *Protéobactéries* sont plus abondantes dans les échantillons de tissus pulmonaires. De plus, en examinant séparément les caractéristiques du microbiote dans les lobes supérieurs (UL) ou

moyens/inférieurs (LL), nous avons identifié des clades entiers avec une abondance inverse entre le LBA et le microbiote des tissus extratumoraux, tels que les *Flavobacteriia* et les *Actinobacteria*, confirmant l'hypothèse initiale de deux communautés microbiennes distinctes.

Étant donné que dans notre plan d'étude, le LBA a été obtenu pour la première fois directement sur un lobe excisé, nous avons eu l'opportunité d'estimer si le LBA des lobes supérieurs était plus semblable à la salive (représentant du microbiote des voies respiratoires supérieures) que celui des lobes inférieurs. Selon la théorie du modèle insulaire adapté de la biogéographie pulmonaire (Dickson et al., 2015), les lobes supérieurs devraient avoir une composition en communautés bactériennes plus semblable à celle des voies respiratoires supérieures. Ils devraient également présenter une richesse accrue par rapport aux lobes inférieurs. Cependant, nous n'avons pas constaté de différence significative dans la distance (Bêta diversité) entre la salive et le LBA du lobe tumoral inférieur ou supérieur, malgré une légère augmentation de l'abondance des taxons typiquement oraux des lobes supérieurs (tels que les genres *Veillonella* ou *Prevotella*). Une explication de ce résultat pourrait être soit la présence de tumeurs qui influenceraient les propriétés du microbiote, comme l'ont suggéré précédemment (Liu et al., 2018), soit à une approche d'échantillonnage différente qui a considérablement réduit la contamination des voies aériennes supérieures.

Nous avons au contraire observé une richesse et une diversité significativement plus faibles dans le LBA des lobes inférieurs. Ces deux éléments sont similaires dans les tissus tumoraux et non cancéreux ; plusieurs raisons sont possibles. Premièrement, le LBA est plus sensible à la distance de la bronche centrale que les tissus, ce qui entraîne une plus faible richesse dans les lobes inférieurs selon l'hypothèse du modèle insulaire adapté à la biogéographie pulmonaire (Dickson et al., 2015). Deuxièmement, comme les lobes supérieurs du poumon sont plus dilatés que les lobes inférieurs pendant l'inspiration lente, une quantité d'air plus importante pénètre dans les lobes inférieurs que dans les lobes supérieurs (Chang & Yu, 1999). Nous pouvons avancer l'hypothèse d'un environnement plus dynamique dans les lobes inférieurs que dans les lobes supérieurs, ce qui limiterait l'établissement d'une communauté bactérienne plus riche, contrairement aux voies aériennes supérieures. Ceci fera l'objet de recherches plus approfondies.

❖ Microbiote des tissus péri-tumoraux comme potentiel marqueur indirect d'une modification de l'environnement tumoral

Il a été démontré que la progression de la tumeur n'est pas seulement liée à la tumeur elle-même, mais aussi à son environnement. Des travaux ont permis d'observer une progression tumorale par remodelage de la Matrice Extra Cellulaire (MEC) sous l'influence de facteurs tumorigènes (Altinay, 2016) (Figure 2). D'autres travaux ont également démontrés les caractéristiques répressives de l'environnement sur la tumeur (Bissell & Hines, 2011). Cependant, aucune étude n'a jusqu'à présent examiné si les tissus péri-tumoraux contenaient des microbiotes qui diffèrent de ceux de la tumeur ou des tissus non cancéreux dans le cas du CBNPC.

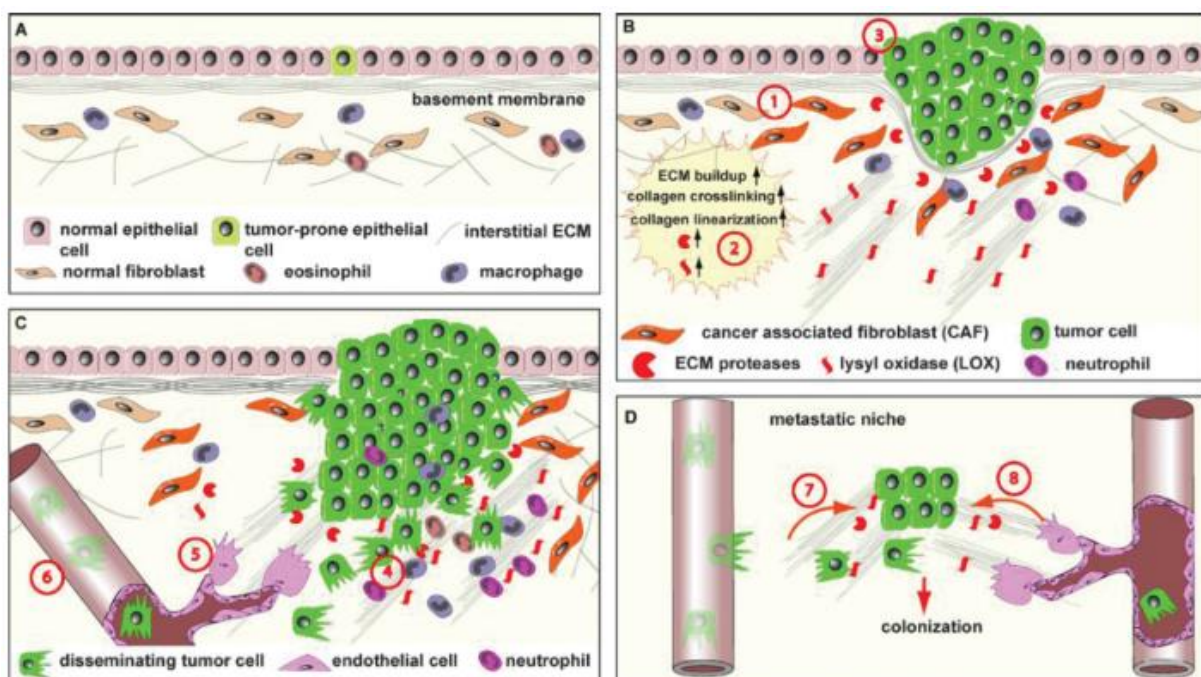


Figure 2 La modification de la MEC favorise la progression du cancer (adapté d'Altinay 2016).

(A) structure normale de la MEC. (B) Dans des conditions pathologiques, il existe une production excessive de fibroblastes activés qui contribuent à la surproduction de MEC et à la dérégulation des enzymes responsables de la modification de la MEC. En raison d'un fragile équilibre entre les cellules locales et la MEC, ces changements stimulent la transformation cellulaire épithéliale et l'hyperplasie, créant une matrice plus rigide. (C) En plus des cellules locales, les cellules immunitaires recrutées sur le site de la tumeur favorisent l'inflammation locale, accompagnée d'une néoangiogénèse facilitant la propagation de la tumeur en raison de la fuite du système vasculaire. (D) Les métastases qui se sont maintenues dans la circulation, s'extravasent au niveau d'un site distal et expriment les enzymes de modification de la MEC pour créer une niche métastatique locale qui favorisera davantage la survie et la prolifération des cellules cancéreuses.

Nous sommes les premiers à inclure le tissu péri-tumoral dans la caractérisation du microbiote pulmonaire (Article 1), montrant ainsi qu'il s'agit du seul échantillon de tissu pulmonaire présentant des changements significatifs de richesse et d'Alpha et Bêta diversité en fonction de la localisation du lobe. Nous avons signalé que les tissus péri-tumoraux des lobes supérieurs

présentaient une similarité accrue avec ceux du LBA (observés en Bêta diversité et avec une présence plus élevée du phylum des *Firmicutes*). Les tissus péri-tumoraux des lobes inférieurs présentent quant-à-eux les mêmes caractéristiques que les deux autres tissus. Nous avons suggéré que ceci reflète en fait différents types de modifications de la MEC, influençant à la fois les propriétés adhésives et l'architecture des tissus (Altinay, 2016; Bissell & Hines, 2011; Quail & Joyce, 2013) et modifiant directement les conditions locales de croissance des bactéries. Dans la pathogénicité du CBNPC, les tumeurs des lobes inférieurs ont été associées à une diminution de la survie globale, mais sans explication claire (Kudo et al., 2012; Riquet et al., 2016). Notre découverte pourrait indiquer que la détection indirecte de différences subtiles dans l'environnement de la tumeur pourraient être liées soit à son agressivité accrue, soit au développement de différents types de microbiotes qui pourraient permettre ou non la progression du cancer (par exemple l'occupation de sites d'adhésion et la progression métastatique).

❖ Lobes inférieurs et présence de ganglions lymphatiques métastatiques marqués par une plus grande abondance du phylum des *Firmicutes*

Plusieurs études ont déjà mis en évidence l'augmentation du phylum des *Firmicutes* dans le microbiote pulmonaire en comparant un groupe témoin à un groupe cancéreux. Cette observation a été d'un intérêt particulier pour le BPCO (Sze et al., 2012) puisque des études ont montré que cette pathologie pouvait conduire au développement du cancer du poumon mais aussi que les deux maladies sont basées sur une inflammation clinique ou subclinique créant ainsi une base mutuelle de « réflexion » (Houghton, 2013; Melkamu et al., 2013).

Nous sommes les premiers à signaler que le microbiote pulmonaire, basé sur l'analyse de quatre types d'échantillons différents, présente une abondance moyenne plus élevée de l'embranchement des *Firmicutes* si la tumeur se situe dans les lobes inférieurs (Article 1). De plus, nous avons observé une augmentation des *Firmicutes* dans les tissus pulmonaires si le patient présentait des changements métastatiques au niveau des ganglions lymphatiques locaux (Article 2). Ces deux résultats concordent avec ceux décrits dans le paragraphe précédent, qui établissait un lien entre ce "déplacement" et l'état avancé de la maladie ou de sa présence. Dans notre étude, ce "déplacement" était principalement dû à une abondance relative significativement plus élevée du genre *Clostridium*. Cependant, la détermination plus précise des espèces impliquées, leur quantité absolue et l'implication de facteurs immuns tumoraux feront l'objet de futures recherches.

❖ Les ganglions lymphatiques métastatiques séparent le microbiote du tissu tumoral et du tissu extratumoral selon un schéma inverse correspondant aux profils respiratoires bactériens

La stadification tumorale est le principal outil de pronostic du CBNPC et sert également au choix du traitement approprié (Goldstraw et al., 2016). Elle est basée sur la classification TNM qui inclut l'information sur la taille de la tumeur, l'implication des changements métastatiques sur les ganglions lymphatiques ipsilatéraux et médiastinaux, et l'apparition de métastases distales. La présence de changements métastatiques aggrave considérablement le pronostic par rapport aux patients présentant des tumeurs plus importantes mais sans changements métastatiques, puisque leur présence a été associée à une survie globale raccourcie de 5 ans (Goldstraw et al., 2016; Planchard et al., 2019).

Nous sommes les premiers à montrer que le microbiote pulmonaire tissulaire présente une configuration spécifique et inverse entre les tissus tumoraux et extratumoraux en fonction du statut métastatique des ganglions lymphatiques (Article 2). De plus, nous avons montré que ce schéma était étroitement lié aux profils respiratoires bactériens. Contrairement aux études précédentes, nous avons pu bénéficier de trois types différents de tissus pulmonaires (non cancéreux, péritumoraux et tumoraux) nous permettant ainsi de mieux caractériser les communautés microbiennes dans et à proximité immédiate de la tumeur. 31 genres bactériens présentaient une abondance significativement différente dans au moins un des tissus entre les patients avec et sans métastases des ganglions lymphatiques. Curieusement, si un genre bactérien détecté dans les tissus extratumoraux était significativement plus abondant chez les patients présentant des ganglions lymphatiques métastatiques, ce même genre montrait exactement des proportions inverses s'il était détecté dans la tumeur (Article 2, figures 2 et 3). Bien que les 31 genres bactériens n'aient pas tous une abondance significativement inverse pour chaque tissu, ils ont tous montré la même tendance à suivre ce schéma général. Une observation encore plus frappante est que les genres bactériens présentant une plus grande abondance dans les tumeurs des patients à ganglions lymphatiques normaux et dans les tissus extratumoraux des patients à ganglions lymphatiques métastatiques avaient tous un profil respiratoire aérobie et appartenaient principalement au phylum des *Protéobactéries*. Inversement, les genres bactériens présentant un schéma inverse avaient un profil respiratoire anaérobie et appartenaient en majorité au phylum des *Firmicutes*. L'observation d'une abondance inverse des profils respiratoires distincts et l'abondance moins fluctuante dans le tissu péritumoral ont conduit à l'hypothèse des bactéries "migrantes". En examinant la physiologie de la tumeur, on constate que les tumeurs plus petites et non agressives sont souvent bien oxygénées, avec un apport

suffisant en sang et en nutriments, tout en offrant une protection accrue contre le système immunitaire de par leur immunogénicité réduite ou à l'immunosuppression (Van Dessel et al., 2015). Les cellules tumorales peuvent également produire différents facteurs qui leur permettent un apport et une administration directe en glucose (Schwartsburd, 2019). Ces conditions pourraient fournir un excellent environnement pour les bactéries aérobies. Cependant, au cours du développement et de la croissance des tumeurs, on observe un taux accru de production d'acide lactique en raison : de son métabolisme anaérobie, de conditions hypoxiques croissantes et d'un apport sanguin insuffisant par des vaisseaux néoangiogéniques défectueux (Fadaka et al., 2017; Wouters et al., 2003). Cet environnement devient par conséquent hostile pour les bactéries aérobies qui se retirent de la tumeur et se déplacent. D'autre part, les bactéries anaérobies, déjà connues pour être présentes dans des biofilms pulmonaires (Costerton et al., 1999; Costerton et al., 1994; Mur et al., 2018; Whitchurch et al., 2002), sont attirées par ce milieu favorable et s'installent ainsi au sein de la tumeur. On pourrait penser qu'elles trouvent dans cet environnement une source de nutriments grâce aux cellules tumorales nécrotiques, tout en étant protégées du système immunitaire par un pH acide inhibant l'activité immunitaire (Damgaci et al., 2018). Même si seulement 25% des genres bactériens détectés ont des organes de motilité actifs, d'autres méthodes de mouvements bactériens provoqués par un stimulus (taxies) ont été décrites précédemment, notamment le "glissement" ou l'attachement aux cellules de l'hôte (Mauriello et al., 2010; Youderian, 1998). Il est donc possible qu'il y ait un échange dynamique entre l'environnement extra et intratumoral en fonction des conditions de croissances variables et qu'il soit d'une grande importance pour notre compréhension de l'interaction tumeur-microbiote.

Le statut métastatique des ganglions lymphatiques est déterminé par biopsie directe, soit avant l'intervention chirurgicale par écho-endoscopie transbronchique, soit durant l'intervention. Dans notre étude, nous avons pu identifier plusieurs biomarqueurs dans chaque tissu pulmonaire qui pourraient indiquer l'implication des ganglions lymphatiques métastatiques sans qu'une biopsie soit directement nécessaire. Cela reste à confirmer par qPCR, technique courante de biologie moléculaire qui pourrait, si elle est validée, faciliter grandement le diagnostic chez les patients atteints de CBNPC.

- ❖ Le microbiote du LBA n'est pas sensible à la présence de ganglions lymphatiques métastatiques, mais il est le plus associé aux phénotypes immunitaires dans le LBA et aux lymphocytes infiltrant les tumeurs

Il a déjà été démontré que l'abondance accrue des taxons supra-glottiques dans le LBA est en corrélation avec l'augmentation de l'état inflammatoire dans les poumons des patients atteints de cancer du poumon. Ceci est observé par le changement du profil Th17 (Segal et al., 2016). Cependant, aucune étude n'a jusqu'à présent évalué l'association entre l'immunité intratumorale et le microbiote pulmonaire.

Nous sommes les premiers à montrer que le microbiote du LBA est associé à la fois au profil immunitaire anti et pro-inflammatoire du LBA et aux lymphocytes infiltrant les tumeurs (TILs) (Article 2). Contrairement aux tissus, le LBA n'a montré aucune différence significative dans l'abondance quant à l'implication des ganglions lymphatiques métastatiques. Cependant, c'est le seul microbiote qui a montré une association significative avec l'immunité locale. Nous avons constaté que les taxons de l'embranchement des *Protéobactéries* étaient positivement corrélés avec les marqueurs protumoraux (Chraa et al., 2019) tels que les neutrophiles activés, les Th17, les Treg dans le LBA et avec une infiltration moindre de la tumeur. D'autre part, la plupart des taxons de l'embranchement des *Firmicutes* étaient positivement corrélés à des marqueurs antitumoraux tels que Th1, Th2, les formes inactives de neutrophiles (Chraa et al., 2019) dans le LBA et avec une infiltration plus élevée de la tumeur. Plusieurs des genres détectés ont déjà été signalés comme ayant une action similaire sur la réponse immunitaire. Par exemple, *Akkermansia*, *Bifidobacterium* et *Lactobacillus* ont été détectés comme des genres bactériens ayant un effet antitumoral (Bashiardes et al., 2017; Naito et al., 2018). Les genres *Haemophilus*, *Streptococcus*, *Staphylococcus* et *Pseudomonas* sont quant-à-eux associés à des pathologies pulmonaires (BPCO) ayant un impact négatif ou immunosuppresseur sur l'hôte (Erb-Downward et al., 2011; Sze et al., 2015).

4.2 Conclusion

Le cancer du poumon est la première cause de décès par cancer dans le monde et il s'agit également du cancer ayant la plus forte incidence en 2018 avec le cancer du sein (International Agency for Research on Cancer, 2019). Avec deux sous-types principaux, le CBNPC représente jusqu'à 80 à 85 % de l'ensemble des cas de cancer du poumon et touche en majorité une population âgée (Takayuki et al., 2018). Bien que sa base génétique soit déjà bien caractérisée

(Aisner & Marshall, 2012), l'interaction entre le CBNPC et le microbiote pulmonaire ne font l'objet que d'études très récentes. Même si la dernière décennie a été marquée par un intérêt croissant pour le microbiote pulmonaire, en particulier le BPCO, l'asthme et la fibrose kystique (Mao et al., 2018; O'Dwyer et al., 2016), le cancer du poumon reste encore peu exploré. Cependant, la situation évolue grâce à de récentes recherches portant sur des modèles animaux, et montrant que le microbiote intestinal peut à la fois influencer la croissance tumorale et la réponse de l'hôte à la chimiothérapie (Iida et al., 2013; Routy, Le Chatelier, et al., 2018; Sivan et al., 2015; Vétizou et al., 2015). Ces résultats évoquent l'idée de l'axe intestin-poumon, l'hypothèse d'une stimulation immunitaire de l'hôte par certaines bactéries intestinales pour augmenter l'activité antitumorale dans les poumons (Bingula et al., 2017; Budden et al., 2016; Hauptmann & Schaible, 2016; Marsland et al., 2015). Néanmoins, avant de considérer l'axe et son effet distal, il a d'abord fallu étudier l'effet local du microbiote pulmonaire (Dickson & Cox, 2017). Les défis ont été multiples, à commencer par l'éthique de l'échantillonnage pulmonaire chez les sujets humains. La majorité des études ont porté sur l'analyse des LBA (Mao et al., 2018). Enfin, de nombreuses questions sont restées sans réponses, comme l'implication que peut engendrer la différence fondamentale entre les différents échantillons pulmonaires dans les conclusions des études menées, ou l'étude approfondie de l'interaction tumeur-microbiote.

Nous avons montré pour la première fois que le microbiote pulmonaire provenant du LBA et des tissus pulmonaires présente des caractéristiques différentes, et qu'ils représentent tous de véritables échantillons pulmonaires en comparaison à l'échantillon de microbiote oral qu'est la salive. De plus, nous avons caractérisé pour la première fois le microbiote de quatre échantillons pulmonaires différents (LBA, tissus non cancéreux, péri-tumoraux et tumoraux) et nous avons également placé nos observations avec la perspective de la localisation du lobe tumoral. Nous avons montré que c'est ce dernier paramètre qui a le plus influencé le microbiote des LBA et des tissus péri-tumoraux, tandis que la tumeur était la moins concernée. Nous avons également avancé l'hypothèse que les changements observés dans les tissus péri-tumoraux pourraient refléter la modification de la proximité de la tumeur et pourrait influencer son agressivité.

Nous avons ensuite montré que l'abondance des tissus tumoraux et extratumoraux est inversement proportionnelle à celle des ganglions lymphatiques métastatiques. De plus, ce schéma était étroitement lié aux profils respiratoires bactériens, identifiant les bactéries aérobies comme étant les plus présentes dans les tumeurs sans ganglions lymphatiques métastatiques et dans les tissus extratumoraux avec ganglions métastatiques. Le schéma inverse a été observé pour les bactéries anaérobies. Nous avons avancé l'hypothèse que ces deux groupes bactériens

se délocalisent selon le microenvironnement tumoral, soit la richesse en nutriments, la protection contre le système immunitaire et des conditions respiratoires favorables. Parmi ces genres bactériens, nous avons identifié des biomarqueurs potentiels qui pourraient contribuer à un diagnostic clinique plus précis et plus facile des métastases ganglionnaires. Enfin, nous avons signalé l'association entre la réponse immunitaire extra et intratumorale locale et le microbiote du LBA, identifiant le phylum des *Protéobactéries* comme étant associés au profil protumoral et le phylum des Firmicutes comme étant associés à un profil antitumoral. Ces observations étaient indépendantes du statut métastatique des ganglions lymphatiques.

Nos résultats sont parmi les premiers à tenir compte du microbiote pulmonaire dans le CBNPC et ils contribuent de façon significative à la compréhension globale de la dynamique locale entre le microbiote, la tumeur pulmonaire et l'immunité. En plus de montrer la différence sous-jacente entre les différents microbiotes pulmonaires, nous avons également démontré la dissimilarité de l'association avec la réponse immunitaire. Nos résultats devront toutefois être confirmés avec un plus grand nombre de sujets, en particulier pour la vérification des biomarqueurs potentiels.

4.3 Perspectives

Bien que nous ayons initié les recherches sur le microbiote pulmonaire et son implication dans le cancer du poumon, de nombreuses questions demeurent sans réponses. Un prochain objectif sera de nous focaliser sur l'effet des ganglions lymphatiques métastatiques sur l'ensemble de l'hôte. Ceci impliquera l'analyse des profils immunitaires (comme déjà décrit dans le LBA), des cytokines dans le sang, et bien sûr, de l'étude des caractéristiques du microbiote fécal et de la production d'AGCC. Dans le cadre de l'application clinique, nous chercherons à confirmer notre découverte de biomarqueurs potentiels en développant la quantification absolue par qPCR, afin de disposer d'une technique rapide et précise pour la détection des ganglions lymphatiques métastatiques à partir d'une biopsie de tissu dans le lobe tumoral.

L'immunologie de la tumeur est devenue un facteur important à prendre en considération dans le choix du traitement approprié ou dans l'établissement du pronostic. Ceci s'explique par le fait que chaque tumeur appartient à l'un des phénotypes essentiels de l'immunité cancéreuse, présentant des caractéristiques spécifiques qui influencent son interaction avec son environnement (Chen & Mellman, 2017). Comme nous l'avons observé dans notre étude (Article 2), il est possible que des numérations très hétérogènes de lymphocytes infiltrant les tumeurs ainsi qu'aucune association avec le microbiote tumoral soit un facteur discriminant. Le

microbiote intestinal sera bien entendu inclus dans l'analyse, puisqu'il a été décrit comme "stimulateur" initial de l'infiltration tumorale par les cellules immunitaires antitumorales dans des modèles animaux (Daillère et al., 2016; Routy, Le Chatelier, et al., 2018; Sivan et al., 2015; Viaud et al., 2013).

En ce qui concerne les métabolites bactériens, nous avons dosé les AGCC dans les fèces, mais selon la littérature, cela ne représente que 5% du total des AGCC produits au niveau intestinal (den Besten et al., 2013). Même s'il s'agit d'un bon indicateur des groupes fonctionnels bactériens et des résultats de dénombrement des selles fraîches, il sera intéressant de doser les AGCC dans le sang. Cela nous permettrait d'avoir un aperçu de la partie absorbée, même si elle est présente en petite quantité (Cummins et al., 1987), qui pourrait atteindre l'environnement de la tumeur pulmonaire et jouer le rôle dans la réponse immunitaire comme suggéré précédemment (Bingula et al., 2017).

Une perspective importante de ce projet est également de compléter le groupe de patients CBNPC qui ont subi un traitement de chimiothérapie avant la chirurgie. Ce groupe nous permettra de suivre l'évolution du microbiote intestinal et salivaire et les marqueurs immunitaires au cours de la thérapie, la chirurgie étant le point final de la collecte des échantillons pulmonaires. Les effets secondaires de la chimiothérapie dus à sa non-sélectivité, comme la perturbation de la muqueuse, des membranes et d'autres cellules à prolifération rapide, ont été l'un des problèmes majeurs de la population traitée (Yu, 2013). Cependant, les patients ne réagissent pas de la même manière, mais malheureusement, la tumeur non plus, comme cela a déjà été démontré (Busch et al., 2016; Catacchio et al., 2018). Chez les modèles animaux, la transplantation fécale de patients ayant répondu au traitement s'est déjà avérée être une solution efficace (Sivan et al., 2015; Vétizou et al., 2015), indiquant que le microbiote intestinal est celui qui influence le plus la sensibilité du patient au traitement. Par conséquent, l'objectif sera également d'essayer d'identifier les différences entre les sous-groupes bactériens présents chez ces patients afin d'essayer d'influencer les non-répondants. Compte tenu de la tumeur pulmonaire et du microbiote pulmonaire, il sera intéressant de corrélérer le degré de réponse tumorale au traitement, comme la rétraction tumorale, l'infiltration immunitaire ou le degré de nécrose, avec les facteurs microbiens pulmonaires et digestifs.

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