UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN

"SCIENZE MEDICHE E CHIRURGICHE TRASLAZIONALI"

CICLO XXXIV

THE AIRWAY MICROBIAL LANDSCAPE OF HEMATOLOGIC PATIENTS AT RISK FOR INVASIVE FUNGAL INFECTIONS: A PROSPECTIVE, OBSERVATIONAL, MULTICENTER STUDY (SNIF)

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ABSTRACT

BACKGROUND AND AIMS OF THE STUDY

Invasive fungal infections (IFI) are opportunistic diseases and the cause of significant morbidity and mortality, especially in haematological patients. The capacity to determine the actual risk for IFI is of fundamental importance to indicate optimal therapeutic choices for patients and individualize therapy to create a balance between the prevention of fungal infection and the occurrence of side effects. The importance of the microbiota, which collectively indicates all the microorganisms colonizing the human body is increasingly being recognized for its role in tissue homeostasis, and alterations in its composition, or dysbiosis, have been associated with a variety of pathological conditions. The airway microbiome is being recognized as an important player in tissue physiology and in protection against colonization by respiratory pathogens, including fungi. Hematological patients are at risk for dysbiotic changes, and it was recently confirmed that patients undergoing hematopoietic stem cell transplantation were characterized by a dysbiotic lung microbiome. In this study we aimed to investigate the possible role of pulmonary microbiota in pulmonary aspergillosis. In particular, the primary objective was the characterization of the lung microbiota in a cohorts of patients susceptible to fungal lung infections due to filamentous fungi, Aspergillus in particular, such as patients with malignant haematological diseases or those undergoing bone marrow transplantation. The secondary objective was the identification of a microbial signature of the airway microbiome predictive of the risk of fungal infection.

PATIENTS AND METHODS

We have designed a multicenter, prospective, observational study called SNIF (Survey of Nasal InFection) in which hematological patients were recruited and their nasal and pharyngeal swabs collected over a 6-month period for microbiome characterization. In order to gain further insight regarding the potential predictive role of the orophayngeal microbiome, each sample was associated with the overall risk of fungal infection of the patient at the time of collection, according to the SEIFEM (Epidemiological Surveillance of Infections in Haematological Diseases)

recommendations. A total of 173 caucasian patients with different hematological diseases recruited from November 2015 to November 2017 from 7 Italian participating centers were enrolled. From each patient, up to six nasal and oropharyngeal swabs were collected over a six-month period. At moment, we focused on pharyngeal swabs.

RESULTS

In a first analysis of the SNIF database, we focused on the oropharyngeal microbiome owing to its higher richness, that may increase the likeliness of uncovering associations with the risk of IFI, for being the main source of the lung microbiota in adults tract and for the potential protective role in pulmonary infections. Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria represented the major phyla accounting for nearly the entire spectrum of bacteria detected in the oropharynx, followed by low-abundant phyla with a cumulative abundance of less than 1%. At the genus level, Streptococcus was found to be the most abundant, followed by Veillonella, Prevotella, Neisseria, Actinomyces, Haemophilus, Fusobacterium, and Rothia, common genera of the pharynx. Upon application of the SEIFEM algorithm, each samples were assigned to low- (LR) and high-risk (HR) groups at the time of collection, respectively. The analysis of alpha and beta diversities revealed that LR and HR samples were associated with distinct microbiota. Indeed, the LR group was characterized by a higher richness and evenness, as measured by Chao1 (LR, median 86, 95% CI 83.1, 88.9; HR, median 51, 95% CI 46.2, 55.9) and Shannon (LR, median 5, 95% CI 4.9, 5.1; HR, median 3.83, 95% CI 3.2, 3.4) indexes, than the HR group . This result was expected as the HR group is associated with a more intense pharmacological treatment that is predicted to impact the microbial diversity more severely. Moreover, the oropharyngeal microbiota of the two groups showed significant differences in compositional structure, as measured by Jaccard (LR, median 0.758, 95% CI 0.757, 0.79; HR, median 0.840, 95% CI 0.839, 0.841) and Bray-Curtis (LR, median 0.718, 95% CI 0.716, 0.719; HR, median 0.825, 95% CI 0.822, 0.827) indexes. Collectively, these results indicate that an algorithm based on demographic and clinical characteristics of patients may provide a useful framework to identify potential signatures of the oropharyngeal microbiome that associate with the risk of IFI. In order to identify signatures of the oropharyngeal microbiome that could be associated with the risk of IFI, we performed high dimensional class comparisons using linear discriminant analysis of effect size (LEfSe) by which we could detect significant differences in the bacterial communities between LR and HR groups. In particular, at the phylum level, the LR group was

associated with a higher predominance of Bacteroidetes, Fusobacteria and Proteobacteria, while Firmicutes were predominant in the HR group. Collectively, these results indicate that microbiome signatures can be identified in oropharyngeal samples of hematological patients that are associated with distinct risks of IFI. To improve the predictive value of microbial signatures we have assessed microbial functional activity in *dynamic*-HR and *dynamic*-LR groups. In particular, genes involved in the biosynthesis of tryptophan (trp), glycolysis and homolactic fermentation were more abundant in HR than LR patients, while genes involved in the fatty acid elongation and the starch degradation pathways (abundantly present in oral bacteria[25]) were more abundant in LR than HR patients . The KEGG database confirmed the association of trp biosynthesis with the HR group. The increased trp auxotrophy would also predict different availability of trp in the HR and LR groups. We measured these levels in a subgroup of pharyngeal samples from 14 consecutive patients undergone HSCT during the study period and found that trp levels were indeed lower in HR than LR patients. Moreover, and importantly, while the levels of L-kynurenine (kyn), resulting from host catabolism of trp, were not different between the two groups, the production of indole-3-aldehyde (3-IAld), an indole that reflects the microbial consumption of trp, was significantly lower in HR than LR patients. These findings suggest an apparent restricted availability of trp for microbial consumption in HR patients. Consistently, amino acid biosynthetic pathways were abundantly present in HR samples Collectively, these results reveal the existence of a transcriptionally active oropharyngeal microbiota that may impact on lung immune status and suggest that not only microbial composition but also active functional activity characterizes the pharyngeal microbiota.

CONCLUSIONS

Overall, these results, while consistent with previous findings in gut, provide evidence for significant differences in oral microbial composition of patients at different risk for IFI. Supported by the high number of patients enrolled, the results clearly highlight the major contribution of neutropenia, the associated antibiotic usage and the occurrence of mucositis in the differences observed between HR and LR patients. As such, these results may pay the way for further studies to uncover associations between the many different risk factors for IFI and the changes in the microbiome. In addition, considering the tolerability and rapidity of the oropharyngeal swabbing, including the advantage of self-collection[211], this sampling method appears highly feasible and relatively at low cost. Thus, if corroborated by further studies, the loss of alpha diversity associated with the loss of

beneficial Clostridiales and Bacteroidetes could help delineate patients at risk of IFI, thereby providing information for antimicrobial therapy optimization. Indeed, HR patients would benefit from an antifungal prophylactic-based approach, as opposed to LR patients, for whom a fungal diagnostic-based approach is recommended to reduce overtreatment and unintended collateral damage to beneficial commensals. Discriminating patients who will benefit or not from antimicrobial prophylaxis will also help reducing the antimicrobial resistance crisis.

INTRODUCTION

Invasive fungal infection (IFI)

Invasive fungal infections (IFI) are opportunistic diseases and the cause of significant morbidity and mortality, especially in immunocompromised patients[1,2.] Hematological diseases include a wide range of malignant conditions and therapeutic strategies employed may vary significantly from one disorder to another [3]. Patients affected by acute leukemia and undergoing allogeneic hematopoietic stem cell transplantation are at higher risk for developing IFI, although other risks factors, including the patients' medical history, changing therapeutic approaches and antifungal treatments, may also play a role in predicting the occurrence of IFI [3]. The highest incidences have been reported in allogeneic hematopoietic cell transplant (HCT) recipients and in patients with acute myeloid leukemia (AML) receiving induction remission chemotherapy [3-8.] In addition, recent studies have shown a high incidence of Infection fungal disease (IFD) in patients with acute lymphoid leukemia (ALL)[9,10], and the emergence of a new group at risk: patients with chronic lymphoproliferative diseases receiving ibrutinib [11-13]. The capacity to determine the actual risk for IFI is of fundamental importance to indicate optimal therapeutic choices for patients and individualize therapy to create a balance between the prevention of fungal infection and the occurrence of side effects [3].

The European Organization for Research and Treatment of Cancer and the Mycoses Study Group Education and Research Consortium (EORTC-MSG) criteria for IFI

It is essential to diagnose the first symptoms as soon as possible, and then create a useful reference model to know the main risk factors and recognize the real early signs of IFI in order to start specific treatment as quickly as possible. First published in 2002, the genesis of the original European Organization for Research and Treatment of Cancer **(**EORTC) and the Mycoses Study Group (MSG) definitions for IFIs arose from the need to have consensus definitions for proven, probable, and possible infection among patients with cancer and recipients of hematopoietic stem cell

transplants[15]. These definitions were established using the combination of host, clinical, and mycologic criteria and were intended for use in epidemiologic and clinical research, but they were adopted by the larger practicing community. The first revision of these guidelines was published in 2008[16]. This revision was necessary in order to address short comings in the original version of the definitions and to incorporate emerging diagnostic methodologies. A key issue addressed in this revision included the elimination of the "possible" category for IFI, as this allowed many dubious cases to be included in the definitions. The first revision also broadened the host population beyond those with hematologic malignancies and stem cell transplant recipients to include solid organ transplant recipients and patients with primary immunodeficiency. The revision also includes definitions for less common IFIs[16]. In the most recent (2020) iteration of these EORTC/MSG Education and Research Consortium definitions, there have been further refinements to this evolving document[17]. The update focuses on 9 topical areas that pertain to hosts, fungal diagnostics, and pathogens. This version of the definitions is limited to the proven and probable categories, as no consensus was reached about the possible category. The host definition now includes the pediatric age group, innate immunologic disorders, and CD4 lymphopenia, among other conditions. Fungal diagnostics continue to evolve with improved sensitivity and specificity, resulting in more rapid and accurate diagnosis. These diagnostics have greatly enhanced the utility of the definitions. Finally, the document now includes definitions for Pneumocystis jirovecii infection[17]. To summarize it all, invasive fungal infections are classified into[14]:

1) PROVEN: In the definite proven infection, the pathogen must be evidenced by a direct histopathological and / or cytological and / or microscopic examination carried out on the tissue sample affected by the fungal infection. It must also have been obtained by a sterile diagnostic procedure such as biopsy or needle aspiration, or isolated by culture from an organic site by definition sterile, such as blood (*Table 1*)
2) PROBABLE: The definition of probable infection is instead based on the presence of at least one factor related to the host, a clinical criteria and a microbiological criterion. combination of three criteria: host factors, clinical criteria and mycological criteria. Probable infections to be defined as such require the coexistence of these 3 criteria (*Table 2*)
3) POSSIBLE: We talk about possible fungal infection in the presence of a host criteria and a clinical criteria, which give sufficient clinical evidence but without mycological support.

Diagnostic tests of IFI

The rate of pre-mortem diagnosis of IFI is in the range of 12–60%. Usually, in clinically unstable patients, IFIs are treated empirically, which raises economic burden, and delay in diagnosis is associated with increased mortality. This indicates the necessity of improved diagnosis[18,19] .Conventional techniques for clinical diagnosis are limited by slow turn around time or associated invasive procedures. Newer methods that do not require detection of the organism in culture or tissues are limited by availability and clinical performance. Thus, standardization of diagnostic tests for each clinical scenario is difficult. The procedures in use are listed below[18]:

Culture-based detection

This remains the gold standard for diagnosis and provides critical data on organism susceptibility. However, it also leads to considerable delays in the initiation of treatment. The time to initiation of antifungal treatment of at least 12 h after the first positive blood culture sample was an independent determinant of hospital mortality in patients with invasive candidiasis[18,20]. A prospective study in 2000 determined that fungal colonization detected by surveillance fungal cultures lacked positive predictive value for diagnosis of fungal infection in critically ill patients, and had minimal utility[18,21]. The sensitivity and specificity of cultures vary for organisms and type of body fluid. Cerebrospinal fluid (CSF) cultures for cryptococcus are highly sensitive and specific, whereas blood culture sensitivity in invasive candidiasis is only 50–60%, with 95% specificity. The positive predictive value of cultures depends on the prevalence of the infection and, therefore, is much higher in transplant patients and endemic areas[18,22] . A study looking at immunocompromised patients with positive lower respiratory-tract culture for aspergillus showed a positive predictive value of 72% in patients with hematologic malignancy, granulocytopenia, or bone-marrow transplant; 58% in those with a solid-organ transplant or using corticosteroids; and 14% in those with HIV infection.[23] Identification of yeast or molds from clinical specimens is exceptionally laborious. Microbiologists have begun using mass spectrometry identification techniques (MALDI-TOF, matrix-assisted laser desorption time of flight mass spectrometry) on specimens for rapid diagnosis.[24]

Histopathology

This is another gold-standard test, especially in invasive aspergillosis, pneumocystis, and endemic fungal infections (histoplasma, blastomyces, and coccidioides), often used in conjunction with culture. Histopathology can detect an invasion of fungus, as well as a host response. For example, visualization of a biofilm can help direct therapy since this is associated with resistance to antifungal regimens. Histopathology, unfortunately, gives only a descriptive diagnosis; fungal morphology can be non-specific, and it does not allow for the identification of species, which is usually necessary to direct therapy.[22]

β-D-glucan

Testing to detect the explicitly fungal cell wall component (1,3)-β-D-glucan (BDG) is commonly used as a screening and diagnostic tool in IFI. BDG is found in several organisms, including Candida species, pneumocystis, and molds such as Aspergillus species and Fusarium species. It is carried out on serum. Values ≥ 80 pg / mL indicate positive test results, while values below 60 pg / mL indicate negative test results.[38] In a prospective study of HSCT recipients, BDG assays were found to have a specificity of 98% and a negative predictive value of 99%, making this test a useful tool to rule out IFI.[26] Similar results were found in a recent meta-analysis in patients with hematological malignancies where two consecutive BDG tests had an excellent specificity (98.9%) but a low sensitivity (49.6%) for the diagnosis of IFI[25]. This low sensitivity is likely related to the higher incidence of colonization in the HSCT population and testing on specific populations like hemodialysis patients or those with Gram-negative bacteremia[18]

Aspergillus galactomannan

GM is another fungal cell-wall biomarker produced by several fungi, including Aspergillus, Penicillium, and Histoplasma species. This assay is reported as an index of optical density (GM index or GMI) and is currently FDA approved for the detection of aspergillus in serum and bronchoalveolar lavage (BAL) specimens. Serum GM testing is now considered the standard of care given strong recommendations for use in immunocompromised patients by several high-quality studies (in patients not on posaconazole prophylaxis)[28]. In a retrospective cohort study in allogeneic HSCT recipients with invasive aspergillosis (IA), the magnitude of GMI (drawn on clinical suspicion) correlated with higher all-cause mortality, likely representing increased aspergillus burden.[29]

Another similar study concluded that a GMI screening strategy could lead to lower mortality by early detection of invasive aspergillosis.[30]

The elective samples for GM analysis are serum and BAL. The concentration of GM is expressed as an index. On serum, a sample is considered positive for values> 0.5 on two consecutive detections or for values> 0.7 in a single sample. The importance of this test lies in the fact that its positivity in serum frequently precedes, by at least 1 week, the development of clinical signs, radiological abnormalities and the growth of the fungus in culture. The search for GM through enzyme immunoassay therefore allows an early diagnosis of IA, which can then impact on what will be the outcome of the fungal infection. The levels of GM detected in serum are also useful for prognosis: very high values at the beginning of antifungal therapy are associated with a worse prognosis; persistent antigenemia, with an index > 1 during therapy is a sign of therapeutic failure; the negativization of the GM test in the first 2 weeks is instead associated with a higher probability of survival. However, one of the problems associated with the use of GM is the possibility of false positives, due to the presence of the molecule in antibiotic preparations such as piperacillin-tazobactam or amoxicillin-clavulanate[25]

Antigen–antibody detection

Sensitivity of antibody tests may be limited in immunocompromised patients, as there is a lack of antibody response to infections. They also have limited specificity, since they cannot differentiate between the presence of normal flora, colonization, and infections[18].

Cryptococcal antigen test

When performed on CSF, this test has a sensitivity of 97% and a specificity of 93–100%. It can also be used on a blood specimen, but it has a sensitivity of 87%. It has become a point-of-care screening test in the immunocompromised population[18].

Histoplasma and blastomyces antigen tests.

These tests can be performed on urine or serum and have superior sensitivities (83.3–91.8% for histoplasmosis and 92.9% for blastomycosis) than antibody testing[18].

Coccidioides antibodies

This is a highly accurate antibody test using complement fixation and tube precipitin antibody detection methods[18].

Molecular studies

Molecular diagnostic methods, especially polymerase chain reaction (PCR), have increased in popularity and replaced some traditional methods due to simplicity of use and rapid turnaround time. The high sensitivity of PCR allows for early detection of infection when treatment is easier and may prevent clinical manifestations. Since quantifying microbial burden in IFI is vital in terms of management, the development of real-time PCR, which can quantify the amount of DNA in real time, has been revolutionary. Some technical difficulties associated with PCR diagnosis include cumbersome methods for cell wall lysis to expose DNA, primer standardization, lab-to-lab discrepancies, and sensitivity to contamination[18,28].

Aspergillus polymerase chain reaction (PCR)

There are several recent meta-analyses analyzing the performance of PCR on blood or serum samples for aspergillus detection. One meta-analysis showed the sensitivity and specificity of blood or serum PCR for two consecutive positive samples were 0.75 (95% CI 0.54–0.88) and 0.87 (95% CI 0.78–0.93), respectively, and for a single positive sample were 0.88 (95% CI 0.75–0.94) and 0.75 (95% CI 0.63– 0.84), respectively.[31] In another meta-analysis that compared the performance of PCR of BAL with a BAL GM test, mean sensitivity and specificity values for diagnosis of proven or probable IPA were 90.2% (77.2–96.1%) and 96.4% (93.3–98.1%), respectively.[32] The American Thoracic Society has published a summary of recommendations based on high-quality meta-analyses for diagnosing IPA in severely immunocompromised patients, such as HSCT with high pretest probability. They recommended testing blood or serum for aspergillus using PCR. In patients with high pretest probability, a single positive PCR gives moderate-to-high sensitivity to exclude the disease, and two positive PCRs provide high specificity. In patients with high pretest probability suspected of having IPA, aspergillus PCR testing of BAL is recommended[27]. However, these recommendations have been made for adult patients only[18].

Candida PCR

Similar studies were performed for assessing the utility of diagnostic PCR in patients suspected of invasive candidiasis. In one meta-analysis of 54 studies that included almost 5000 patients tested

by blood-based PCR, pooled sensitivity and specificity for proven or probable invasive candidiasis versus at-risk controls were 0.95 (CI, 0.88–0.98) and 0.92 (CI 0.88–0.95), respectively.[33] Recently, there has been an innovative contribution to invasive candidiasis diagnostics in the form of targeted molecular direct detection using an FDA approved candida nucleic acid test[18].

Aspergillus PCR + galactomannan

An emerging strategy is to combine molecular and antigen testing to improve clinical utility. This was assessed in a recent meta-analysis of high-risk hematological patients, which found that the highest sensitivity (99%) was achieved when at least one positive GM or PCR was used to define a positive episode. The absence of any positive test result had a negative predictive value of 100%, averting unnecessary antifungal exposure[18,34].

PCR + BDG assay

A similar approach was made for the diagnosis of Pneumocystis jirovecii in a large HSCT population with clinical suspicion of P. jirovecii pneumonia (PJP). This retrospective cohort study used PJP PCR on bronchoscopy samples and serum BDG testing. In patients with a positive BAL PCR result, a positive BDG resulted in 100% specificity with a 100% positive predictive value[35]. Given the high mortality related to invasive fungal infection, prophylaxis and empiric therapy should not be delayed while awaiting identification of the pathogen[18,36].

Epidemiology

Aspergillosis, candidiasis, fusariosis, mucormycosis, cryptococcosis and trichosporonosis are the most important infections reported in patients with hematologic malignancies (HM) that undergo hematopoietic stem cell transplantation. These infections are caused by opportunistic fungal pathogens that do not cause severe issues in healthy individuals, but in patients with hematologic malignancies lead to disseminated infection with different clinical manifestations[34].
The epidemiology of fungal infections has undergone a profound change in recent decades, showing an increasing incidence of mold infections (filamentous fungi), such as especially Aspegillus spp and Zigomicetes, with a reduction instead of yeast infections (unicellular fungi), such as for example Candida spp[7.]

Aspergillosis

Invasive infection of humans is most frequently caused by members of the Aspergillus fumigatus complex, followed by A. flavus, A. niger, and A. terreus. A. fumigatus is most common in the lung, whereas A. flavus more commonly causes infection of the larger passageways and sinuses. In contrast, burn wounds are commonly colonized by A. niger and A. flavus[36,46]. The pulmonary system is exposed to aspergillus conidia daily, and a highly coordinated immune response has evolved for rapid pathogen elimination. The proximal airways remove conidia through mucociliary clearance, and if this process is impaired (e.g., in cystic fibrosis and bronchiectasis), colonization or infection may develop. Airway epithelial cells and alveolar macrophages are the first line of defense against potential aspergillus infection. They must kill phagocytosed conidia while minimizing the surrounding inflammatory reaction and maintaining immune homeostasis. The bronchial epithelium can internalize conidia; however, hyphae are able to pass through the epithelium without disturbing its integrity.[37] Dectin-1, DC-SIGN (dendritic-cell–specific ICAM 3 [intercellular adhesion molecule 3]–grabbing nonintegrin), and pentraxin 3 have been identified as key macrophage receptors assisting in the recognition and phagocytosis of these conidia.[38,39] Polymorphisms in the host genome in these sites and others have been found to predispose patients to invasive aspergillosis.[40] After phagocytosis, killing occurs through generation of NADPH-dependent reactive oxidant species (ROS). Patients with defects in this pathway (e.g., those with chronic granulomatous disease) have invasive infection with aspergillus and other pathogens. Additional signaling pathways regulating aspergillus immunity have also been identified recently, although they are incompletely characterized. These include the calcium–calcineurin–NFAT (nuclear factor of activated T cells) pathway, which is disrupted by calcineurin inhibitors commonly used during the care of patients who have received stem-cell or solid-organ transplants, and Bruton’s tyrosine kinase inhibitors such as ibrutinib, which is increasingly used in patients with lymphoproliferative cancer. Neutrophils have long been recognized as the most important immune cell with activity against aspergillus[46]. Neutrophil recruitment depends on chemokine release from lung epithelial cells[41] and CARD9 (caspase recruitment domain–containing protein 9) signaling, and defects in this latter pathway lead to extrapulmonary aspergillosis. The process of recognition is similar to that for alveolar macrophages and downstream NADPH oxidase–induced ROS production, causing fungal cell death. Host neutrophils release antimicrobial peptides (e.g., defensins) and proteases and attempt to sequester iron availability in response to fungal invasion.[42] T cells are also essential in the host defense, with both CD4 and CD8 cells providing protective immunity.[43] Chronic non invasive forms

of aspergillosis, such as asthmatic exacerbations, allergic bronchopulmonary aspergillosis, and chronic pulmonary aspergillosis, are also defined by aberrant T-cell responses. A dominant type 2 helper T-cell response is observed in allergic diseases, whereas a proinflammatory phenotype has been described in chronic forms of pulmonary aspergillosis[46]. The spectrum of clinical presentations of aspergillosis is defined by the site of involvement: Allergic bronchopulmonary Aspergillosis, Aspergilloma, Cutaneous disease, Tracheobronchitis, Chronic pulmonary aspergillosis, Sinus disease, Disease of the central nervous system and Invasive infection[46]. The severity of invasive infection correlates inversely with the immune status of the host. A high index of suspicion is required for the diagnosis of invasive disease, since an immunocompromised patient may be relatively asymptomatic, precluding early diagnosis. Imaging is a critical component in the diagnostic evaluation of pulmonary and sinus infections in particular. Radiographically, computed tomographic imaging of the chest shows focal consolidations, and in the case of invasive pulmonary infection, consolidation may be characterized by nodules with surrounding ground glass infiltrates (halo sign) or cavitating lesions. Patients with tracheobronchial infection may have no parenchymal lung changes, but debris may be visible in large airways. Non invasive disease, such as allergic bronchopulmonary aspergillosis, is suggested by central bronchiectasis or parenchymal opacities and prompts testing such as measurement of IgE levels and examination of the peripheral-blood eosinophil count[46]. Central nervous system lesions[44] are more common in patients with an underlying genetic immunodeficiency[45] and are seen with immunosuppressive therapy that targets the pathways involved[46].

Risk Factors of IFI

The identification of risk factors predisposing to IFIs in HMs may be extremely complex in clinical practice. The patients' medical history, including the home environment, previous lifestyle, actual HMs and disease stage, and the role of leukocytes are still of great significance in predicting the onset of IFIs[3,48,49,50] . Moreover, in the era of new drugs, a great deal has changed in terms of therapeutic approaches and antifungal treatments. On one hand, a growing number of patients is being treated with chemotherapy-free regimens with a prevalent immunomodulating action[3,51,52] .On the other hand, the introduction of mold-active antifungal prophylaxis (i.e., posaconazole or voriconazole) has changed the epidemiology, clinical and laboratory manifestation and timing of fungal infections[3,53] Lastly, the role of the lung microbiome in the pathogenesis of invasive aspergillosis remains unclear but is the subject of ongoing work. Microbiome mediated mechanisms

of resistance and alteration of the host immune response may increase fungal colonization rates and cause infection during periods of immunosuppression.[47] The effects of mycoviruses or other viruses on aspergillus infections also have yet to be determined[46].

Risk factors in acute myeloid leukemia

Acute myeloid leukemia (AML) is the hematologic disease with the highest rate of IFIs, with an incidence ranging from 10 to 25% according to SEIFEM epidemiologic studies[3,7,54-56] The overall outcome of AML has improved in recent years, mainly thanks to improvements in supportive care, in particular introduction of new antibiotics and use of new diagnostic methods of IFI. For this reason, although the incidence of the IFIs is still very high in AML, particularly during the remission induction phase, the IFI-attributable mortality has decreased progressively, going from 60 to 70% in the past to the current 20–30%[49,57-59]. However, AML is a very heterogeneous disease, and the incidence of IFIs is highly variable depending on the type of leukemia, the patient's characteristics and the fungal exposure[49,57-63]. For example, acute promyelocytic leukemias (APLs) have a documented lower incidence of IFI complications than other AML subtypes, probably due to the mild induction chemotherapy and the short duration of severe neutropenia. Indeed, patients with APL receiving a chemotherapy-free treatment could be considered at low risk for IFIs[3]. In a prospective epidemiologic study by SEIFEM (including more than 1000 AML cases), the following pre-treatment variables were identified in multivariate analysis as high risk factors of IFIs after the first course of chemotherapy: performance status of 2 or greater; chronic obstructive pulmonary disease; recent house renovation; and job with high exposure, such as construction work, farming and gardening [50,57].Overall, the risk factors for IFIs in AML can be classified in four main categories: leukemia-related factors (advanced stage of the disease, failure to enter CR), host-related factors (performance status, comorbidities, older age, organ dysfunction, unfavorable genetic pattern), treatment related factors (deep and prolonged neutropenia, severe mucositis associated chemotherapy), and fungal exposure-related factors (patient rooms without HEPA filters, previous IFI). These factors are reported in *Table 3*[3,50,57,59,61]. The definition of risk factors for IFI might allow the identification of three main groups of AML patients (High risk, Intermediate risk and Low risk) and contribute to designing their diagnostic, prophylactic and therapeutic approaches. Indeed, risk stratification may be considered a useful tool for defining high-risk patients who might benefit from avoiding the overtreatment of low-risk patients. A careful assessment of pre- and post-treatment risk factors for IFIs should become part of our routine evaluation of patients at the time of the

diagnosis of AML and over the course of the disease [49,57,61,62]. A delay in bone marrow blast clearance after induction chemotherapy along with additional risk factors contribute to favor infection complications[57.] This so-called "dynamic adapted antifungal strategy" may enable clinicians to select the best patient-tailored antifungal strategy and may improve the management of IFIs in all phases of AML [57,61] .

Risk factors in Myelodysplastic syndromes

Myelodysplastic syndromes (MDSs) are associated with a risk of severe infections due to quantitative and qualitative granulocytic defects, such as impaired bactericidal and fungicidal activities; reduced expression of the CD11b/CD18 complex; and functional anomalies of myeloperoxidase, lysozyme, superoxide anion lactoferrin and antibiotic proteases such as elastase and cathepsin G [64,65]. Other immunological abnormalities include impaired B, T, T-reg and NK (NK G2D) cell functions [66]. In addition, advanced age, the presence of comorbidities and iron overload are significant additional risk factors for MDS. Iron is an essential factor for both the growth and virulence of most microorganisms. Iron overload, which is frequently observed in MDS due to red blood cell transfusions, increases the risk of bacterial infections and IFIs, such as mucormycosis or aspergillosis, through complex mechanisms, including the inhibition of IFN-gamma, TNF–alpha, and IL-2 and the impairment of macrophage, neutrophil and T-cell functions [3,66-69]. In some prospective registries of IFIs, the incidence of proven/probable IFIs in MDS is lower than that reported in AML [70-73]. Patients with transformed MDS can be treated with either AML-like chemotherapy protocols or hypomethylating agents. In a prospective multicenter observational study on decitabine treatment in 101 MDS patients (47.5% high-risk), the rate of infectious events was significantly higher during the first 3 courses, with an IFI incidence of 12% during 97 febrile episodes[74]. In another retrospective multicenter study in 157 high-risk MDS patients treated with azacitidine, the incidence of IFIs was 4.8%; in univariate analysis, the most important risk factors for infections were low hemoglobin level, low platelet count, unfavorable cytogenetics and low neutrophil count; additionally, in this study, the rate of infections decreased gradually along with the progression and probable efficacy of therapy[75] . In contrast, the risk of IFIs significantly increased in MDS patients treated with azacitidine as salvage therapy after intensive chemotherapy (IC) compared to patients who received front-line azacitidine (risk difference of 22.4%) and in those treated with azacitidine at a standard dose (75 mg/m2 for 7 days) compared to short-schedule treatment (75 mg/m2 for 5 days) [76,77]. Data reported in these recent clinical trials indicate that the most relevant risk factors of

IFIs in MDS patients receiving hypomethylating agents seem to be: 1) High IPSS risk 2) Type of azacitidine treatment (salvage after IC or conventional dosage of 75mg/m2 for 7 days); and 3) Number of azacytidine or decitabine cycles, with a higher risk during the first 2–3 cycles[3].

Risk factors in Acute lymphoblastic leukemia

The risk of developing IFIs in acute lymphoblastic leukemia (ALL) patients has not yet been fully elucidated. Some retrospective studies also demonstrated a not irrelevant IFI incidence in ALL patients, which was 6.5% in the SEIFEM study [7] , with a predominance of mold infections (4.3%), particularly during induction/reinduction treatment. There are some discrepancies regarding the incidence of IFIs in two different studies on prophylaxis in a well-defined setting, such as in acute leukemia (AL) induction patients. In a randomized (caspofungin vs. investigator's choice) prospective study in AML/ALL induction patients, only one case of proven/probable IFIs was reported among 37 patients (2.7%)[78] ; in a larger study comparing liposomal amphotericin B to placebo, the percentage of IFIs was higher, with a 7.9% and 11.7% incidence in the two arms, respectively. This high incidence may reflect a more aggressive schedule of treatment given to adult ALL patients, which was recently introduced with the aim of improving the percentage of long-term survivors; still, this value is disappointing if compared to observations made with a pediatric population[79]. Indeed, the more intensive, pediatric-like schemes demonstrated better results in younger adults, with a low incidence of infections [80,81], while this approach exhibited elevated toxicity, mainly due to infectious complications during induction treatment, among older ALL patients, resulting in lower event-free survival (EFS) and overall survival (OS) [82]. High doses of dexamethasone were also associated with a relevant incidence of IFIs during the induction phase[3]. For example, cases were reported in elderly patients in the phase 2 GRASPAAL/GRAAL-SA2-2008 study (23% IFIs during induction phase 1) [83]. Few studies reporting data on IFI incidence are available in relapsed/refractory ALL, which is considered a category at high risk for infections. IFIs were responsible for death in 6.5% of relapsed/refractory ALL patients in the PETHEMA group study[84]. Tyrosine kinase inhibitors (TKIs) significantly improved the outcome of Philadelphia-positive (Ph+) ALL. Infectious complications due to IFIs were relatively low when TKIs were associated with standard or reduced-intensity chemotherapy as a first-line treatment (3% and 3.8% in the PETHEMA and NILG studies, respectively) [85,86]. Few data are available on the incidence of IFIs during monoclonal antibody-containing regimens or new treatment options, such as blinatumomab or chimeric antigen receptor (CAR) T-cell therapy. Although cases of fatal Candida spp. infections have been reported in relapsed

patients treated with blinatumomab[87]. ALL can be considered a risk factor for IFIs in elderly patients, particularly those over 55 years, receiving intensive (pediatric-like) induction therapy or reinduction for relapsed ALL including high cumulative doses of corticosteroids. Younger adults, patients in complete remission and those receiving less intensive regimens, including TKI inhibitors, are associated with a low risk for IFIs[3]. Adverse biological features may also be helpful in the early identification of a proportion of poorly responsive ALL patients who should be considered susceptible to IFI[57].

Risk factors in Chronic lymphoproliferative disorders

In chronic lymphoproliferative disorders, the incidence of IFIs varies from 0.5 to 10.8% and seems to have increased in the last few years, probably due to more widespread use of new targeted treatments. For non-Hodgkin Lymphoma (nHL), from 2005 to 2015, 7800 patients have been enrolled in 7 prospective[61,88] and 5 retrospective[7,89,90] studies. The incidence of IFIs rose from 1.6% in 2006 [7] to 4.3% in 2014–2015[89]. The average incidence was 2.6%. Risk factors were analyzed in several studies [61,88,89], and multivariate analysis showed that severe and prolonged neutropenia, the status of the disease (advanced versus the diagnosis), and prior IFI were factors independently associated with the occurrence of IFIs[3]. The incidence of IFIs patients with Hodgkin's Lymphoma (HL) ranged from 0.3%[91] to 1.2% [89], although no definite risk factors were identified, except severe and prolonged neutropenia [88,89].Consequently, patients with HL may not be considered at risk for IFIs, and in this setting, screening for IFIs should not be performed routinely, but only when clinically required[3]. However, particular attention must be paid when patients receive very aggressive treatment[90]. For multiple myeloma (MM), the incidence of IFIs ranged from 0.4% to 14% in the most studies[89,91-94]. The multivariate analysis of risk factors identified severe neutropenia, use of bortezomib, three or more lines of treatment and a previous history of IFI as the main factors affecting the occurrence of IFIs [3,61,93,94]. For chronic lymphocytic leukemia (CLL), the incidence of IFIs ranged from 0.5%[57] in the early 2000s to 7.8% in the recent study [90]: univariate analysis showed that neutropenia, prior IFI, lymphocytopenia, the stage and state of the underlying malignancy, CD38 expression, genetic analysis (p53, ATM or 12+), and IgVH mutation status were all factors associated with the presence of IFIs. In all the other chronic lymphoproliferative disorders, severe and prolonged neutropenia, the stage and state of the underlying diseases and more than two therapeutic lines were the most important risk factors for IFIs when multivariate analysis was considered[3].

Risk factors in Myeloproliferative neoplasms (MPNs)

Myeloproliferative neoplasms (MPNs) include chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis (MF). Data regarding the epidemiology of infectious complications in MPN are scanty and mainly related to outdated treatment modalities[3]. Recently, the availability of new targeted drugs has significantly modified the therapeutic landscape in MPNs, improving survival and disease-related symptoms [95-96]. However, some concerns regarding the immunosuppressant activity of these drugs were raised after the documentation of opportunistic infections during treatment [97]. Most patients affected by CML are diagnosed in the chronic phase (CP); a minority may present after progression to the blastic phase (BP), which is comparable to acute leukemia. The survival of CML patients has been dramatically improved with the availability of TKIs targeting the BCR-ABL1 oncoprotein, leading to disease control in the great majority of patients. In-vitro studies have demonstrated that tyrosine kinase inhibition affects the cell-mediated immune-response, possibly creating a permissive microenvironment for opportunistic infections[98.] Additionally, a non-negligible rate of neutropenia is observed during treatment, especially during the first months of therapy. Despite these relevant findings, registrative trials, IRIS (imatinib vs. interferon plus low-dose cytarabine), DASISION (dasatinib vs. imatinib), ENESTnd (nilotinib vs. imatinib), BELA (bosutinib vs. imatinib) and PACE (ponatinib) did not report IFIs in CP CML patients [99-102]. The majority of currently approved kinase inhibitors are significantly affected by CYP3A4 inhibitors/inducers. Although fungal infections are uncommon in patients with CML, caution is required when TKIs are used with azole antifungals, which are moderate or strong CYP3A4 inhibitors[3]. ET and PV are chronic pro-thrombotic diseases with favorable prognosis and no increased incidence of infections. Conversely, infections are one of the main causes of morbidity and mortality in MF, with approximately 10% of patients dying from infections and sporadic cases of fungal complications[103]. The increased infectious risk in MF depends on intrinsic immune deregulation but also on treatment strategies [105] . Targeted therapy with JAK (Janus kinase) inhibitors has shown promising activity in controlling constitutional symptoms and splenomegaly in MF and PV. Ruxolitinib, the first approved JAK1/JAK2 inhibitor, was recently associated with the occurrence of opportunistic fungal infections, namely Cryptococcus neoformans and Pneumocystis jiroveci pneumonia, nodal and lung involvement by Talaromyces marneffei and sino-orbital mucormycosis [3,106]. A multicenter Italian study in 507 MF patients on infectious complications in MF reported 112 cases of grade 3–4 infections. Among these complications, only 2 cases of IFIs were detected[106]. In that cohort, disease status in terms of IPSS risk score and massive splenomegaly were

found to correlate with an increased risk of infection, but this effect was not specific for IFIs. Overall, fungal infections represent a rare but potentially fatal complication in MF. No evidence has been found of specific risk factors for IFIs in these subsets of patients[3].

Risk factors in Autologous stem cell transplantation

Overall, the incidence of IFIs in patients receiving ASCT for HMs ranges from 3% to 8% [6,7,8,92,107]. Data from the literature do not allow us to fully understand there reasons for the great variability of IFI incidence reported in the last decade. Most published articles are based on retrospective studies, and only a few of them included a consistent high number of patients with lymphoma and myeloma[92]. Along this period of observation, an apparent reduction of the mortality correlated with IFIs has been reported, as has a prevalence of mold infections[6,7] 6. The local epidemiology or the specific antifungal use in different centers may have an impact on the incidence of IFIs. However, some independent risk factors emerged from the reported studies, including prior fungal infection, Candida colonization, the duration of neutropenia, the duration of steroid treatment, the use of fludarabine and the advanced status of disease [92,107,108]. To date, there is not stringent evidence that either the prior use of fludarabine ASCT in patients with lymphoma or the use of IMIDs in MM patients may induce an increased risk of fungal infections[3].

Risk factors in Allogeneic hematopoietic stem cell transplantation

Allogeneic HSCT recipients represent one of the categories of patients at high risk of developing IFIs [49]. According to the most recent epidemiological studies including a large number of patients, the reported incidence of IFIs ranges between 7% and 15% [8,107,109].Nevertheless, there is now ample evidence showing that vulnerability to IFIs appears to be multifactorial, including standard, well-known clinical risk factors and other new factors that can impact antifungal defenses. It is of utmost importance to discriminate between risk factors already present at the time of HSCT and unpredictable variables that might occur during the post-transplant clinical course[3].-Age is a well-recognized risk factor, even among allogeneic HSCT recipients, although a specific threshold has not been defined [7,110,111]. There is no doubt that the patient's history, the type of underlying malignancy (MDS/AML, lymphomas) and the presence of active hematologic–disease will certainly predict vulnerability to infection during conditioning and transplantation[5,122]. Iron overload (IO) has been identified as an independent risk factor for invasive aspergillosis [3,113,114], although two major drawbacks limit its applicability in the clinical practice. First, the estimation of the iron burden is

primarily based on serum ferritin as a surrogate for IO; however, many confounding factors, particularly in HSCT recipients (GVHD, liver damage, inflammation), may result in potential ferritin overestimation. Second, a specific threshold of serum ferritin defining the risk for IFD has not been identified. A consistent number of studies have documented that patients receiving transplants from alternative donors are at a high risk for IFIs, while those receiving grafts from matched sibling donors in the absence of additional risk factors should not be considered a risky procedure[3,5,7107,112]. The presence of polymorphisms in genes such as TLR-4, dectin-1 or pentraxin have been reported to significantly-influence the occurrence of post-HSCT IFIs when associated with high risk transplants (MUD, haplo), although it should be emphasized that a large number of potential genetic risk factors for IFIs have been described[38,115,116]. We know that neutropenia is no longer the only primary risk factor for IFIs after HSCT. In fact, many IFIs develop when neutrophil counts have been normalized, months or even years after the transplant, when abnormalities in lymphocyte counts and functions remain the main risk factor [3,117,118]. The crucial role of GVHD and immunosuppressive treatments (ISTs) in the development of IFI has been documented by several studies[5,109,110,111]. In this respect, the Seattle group showed that patients with moderate-to-severe GVHD who were treated with high-dose corticosteroids had a significantly increased incidence of IFIs[119]. Corticosteroids compromise the neutrophil and monocyte– macrophage activity as well as immunity to fungi by inducing lymphopenia, decreasing lymphokine production and inducing Th1/Th2 dysregulation. Similarly, the use of other ISTs, including basiliximab, alemtuzumab, ATG and infliximab, dramatically increases the rate of IFIs[107,109,120,121]. CMV infection is a well-documented predisposing factor for IFI in allo-HSCT. In fact, CMV it self modulates the immune response by suppressing the function of antigen-specific CTLs and by impairing neutrophil activity and macrophage respiratory burst. Notably, treatment of CMV infection commonly includes ganciclovir, which in turn may be considered an additional worsening factor due to the drug related neutropenia[122]. Lastly, high environmental Aspergillus spp. spore counts represent-a significant risk factor for IFI in HMs and particularly among HSCT recipients[123]. Taken as a whole, the defective recovery of both innate and adaptive immunity after HSCT may be considered as a condition shared by all risk factors, ultimately favoring the development of IFIs[3].

Risk stratification for invasive fungal infections in patients with hematological malignancies: "DYNAMIC RISK SCORE" according SEIFEM recommendations

More recently, an Italian study[3] in patients with HM demonstrated that the risk factors of infection described above change from day-to-day. In agreement with other recent reports, at present, the risk stratification for IFI should take into consideration the "non-static level of risk" for IFI. For instance, the risk of IFI could be low in patients at the time of diagnosis of the underlying hematological malignancy, while in the following months, during the management of HM, the same patient could be considered at high risk in the case of non-responsiveness to the anti-neoplastic treatment. Therefore, it is possible to use a "dynamic risk score" for all categories of HM based on epidemiological data, new and old risk factor (patients related and disease related) and expert opinion. This risk stratification could be considered at present the most reliable to evaluate the potential risks for IFIs in patients with HMs according diagnosis, phase and type of treatment[3]. All hematological patients can be divided according risk infection into three groups: HIGH RISK, INTERMEDIATE RISK and LOW RISK. In particular, Acute Myeloid Leukemia (AML) patients undergoing induction chemotherapy with any risk factors (neutropenia at baseline, low complete remission probability, age > 65 yrs, significant pulmonary disfunction) and those with AML who had a prior fungal infection or refractory AML or Myelodysplastic syndromes (MDS)/AML in azacitidyne therapy after intensive chemotherapy as a salvage regime or HSCT (from donors other than a matched sibling donor, patients active HM, GVHD requiring high-dose steroids and history of previous IFI) or Acute Lymphoblastic Leukemia (ALL) patients with any risk factors (age > 55 yrs, intensive regimens, HD dexametazone) or ALL relapsed/refractory are at high risk. On the other hand, AML patients undergoing first remission induction/consolidation therapy without risk factors for IFI, Acute Promyelocytic Leukemia (APL) treated with ATRA/ATO, Myeloproliferative neoplasms (MPNs), Myeloma Multiple (MM), Non-Hodgkin's Lymphoma (NHL), Hodgkin's Disease (HD) treated with conventional chemotherapy, ALL without risk factors (younger adults < 30 yrs, undergoing maintainance treatment for complete remission or with TKI + steroids therapy) are at low risk. Patients undergoing autologous trasplantation, MDS during the first 2–3 cycles of Azacitidyne/Decitabine, MM-NHL-HD refractory or > 3 lines of chemotherapy, HSCT (from matched sibling donors, patients in complete remission with no evidence of GVHD and no previous IFI) are at intermediate risk. Until now, this possible algorithm is the best method for stratification of risk of infection in different hematological diseases as shown *in Table 4.*

The Definition of microbiota

The term microbiome was first used by Lederberg and McCray “to identify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space”[124]. In 2015, based on the concept of “biome”, i.e., the biotic and abiotic factors of a given environment, Marchesi and Ravel, defined the microbiome as the entire “habitat” that is comprised of the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e., genes), and the surrounding environmental conditions[125]. Humans co-evolved with microbes in the environment and each body habitat has a unique set of microorganisms in its microbiota, which is established within the first 1–3 years of life and remains relatively stable throughout the life span [126]. Residential microbes perform metabolic functions and are involved in host functions such as defence, metabolism, and reproduction[127] . The microbiota usually was defined using molecular methods relying predominantly on the analysis of 16S rRNA genes, 18S rRNA genes, or other marker genes and genomic regions, amplified and sequenced from given biological samples[125]. Taxonomic assignments can be performed using a variety of tools that assign each sequence to a microbial taxon (bacteria, archaea, or lower eukaryotes) at different taxonomic levels according to phyla, classes, orders, families, genera, and species[125]. In each body district, only a few phyla are represented, accounting for hundreds of bacterial species[128]. Conserved genes are a target that is used to study the microbiome, particularly in bacteria. At present the most used molecular method to study the microbiome is sequencing of regions of a conserved gene, such as the hypervariable regions of the 16S ribosomal RNA gene[129]. Other higher resolution methods for microbiota profiling are currently being used to disclose the functional link between the lungs and its microbiota [130].

The Lung Microbiota

Although originally believed to be sterile, the lung shows a microbiota that varies in both physiological and pathological conditions[131]. Furthermore, bacteria, fungi, viruses, and their interactions may all be important in lung health and in the development of respiratory diseases[132]. The finding that a unique lung harbours a unique microbiota, irrespective of health or disease, has prompted a wealth of research to not only categorise the distinct microbiota of the healthy lung, but also that of the sickly lung[132]. The healthy human lung contains a variety of commensal microorganisms, especially bacteria, that can show substantial heterogeneity between individuals and across the different lung regions[133]. The Lung microbiome (LMt) composition differs significantly

between the upper and lower respiratory tracts in healthy individuals, enquiring if samples of the upper airways can reflect the microbiome in the lower respiratory tract [134]. The prevalence of distinct bacterial species in these compartments suggests the hypothesis of niche-specific microbial colonization at distinct anatomical sites[135]. Many authors agreed that the healthy LMt has a low density of microbial populations, mainly represented by phyla such as Firmicutes, Bacteriodetes, Proteobacteria, Fusobacteria, and Actinobacteria[136]. Mathieu et al. [137] hypothesized that small numbers of bacteria maybe critical for good health, but which microbial genera constitutes a "healthy microbiome" per se remains hotly debated. Both anatomical characteristics and breathing patterns seem to influence the access of microbes to the lungs[137]. Although the lungs are subjected to constant immigration from the oropharynx, their microbiome is distinctive to that of the upper respiratory tract which, in turn, displays greater similarities to that of the stomach[138]. Dickson et al. [139] proposed three models to explain maintenance of microbial homeostasis in health the modalities with which the homeostasis may be lost in disease. The first model postulates that the community of species forming the LMt is determined by the balance of three factors: migration, elimination, and reproduction rates of microbiota as determined by regional growth conditions[139]. Migration to the LMt is at least in part attributable to micro-aspiration that has long been known to be frequent even among healthy subjects[139]. Bacteria elimination is commonly due to the mucociliary clearance, as well as the innate and adaptive immune defences. According to Dickson's conceptual model[139], factors involved in the reproduction rates of LMt include oxygen tension, pH, blood perfusion, alveolar ventilation, temperature, and the concentration and activation of host inflammatory cells. The second model implicates the availability of nutritional factors as a key determinant of all reproducing bacterial communities. Since in healthy conditions, the airway lumen mainly contains air, the availability of nutrients for most bacteria is relatively limited[140]; this fact might account for the minor role of local reproduction in the composition of the bacterial community in healthy subjects. On the other hand, the airways of patients with obstructive airway diseases, such as cystic fibrosis, chronic bronchitis, bronchiectasis, and asthma, contain a dense, protein-rich growth medium of secreted mucus[141]. Furthermore, in some clinical conditions, such as pneumonia and acute respiratory distress syndrome (ARDS), the alveoli are flooded with protein-rich edema from an injured alveolar–capillary barrier. All these environments may contribute to the bacterial overgrowth and microbiota changes during lung diseases. The final proposed mechanism is the so-called "signaling stress response", a molecular mechanism by which tissues and cells reciprocally communicate perturbations of the internal "milieu". Signaling molecules include hormones (e.g.,

glucocorticoids, estrogens, and androgens), neurotransmitters (e.g., catecholamines and endogenous opioids) and cytokines (e.g., TNFs, IL-1, IL-6, and IL-8) [142,143]. Recent data documented that some microbes can identify and adapt to the signaling molecules that human cells use to communicate[139,144].

The intestinal microbiota and the gut–lung axis

The connection between the lung and the gastrointestinal tract is not entirely understood. Patients with respiratory infections generally have gut dysfunctions complications, which are related to a more severe clinical course, thus indicating gut-lung crosstalk. A study concerning cystic fibrosis provides evidence that the gut microbiota and the lung microbiota develop at the same time after birth, and it is evident that there is a constant crossed dialog between these two areas [145]. A certain number of bacteria appear in the gut before being detected in the respiratory tract; this indicates a contribution of micro-aspiration of gut microbes in the development of the microbiota of the airways. The bacteria producing lactic acid and Lactobacilli, in particular, increase the activity of the natural killer cells, increase the antiviral immune response, reduce the production of pro-inflammatory cytokines and determine an up-regulation of the cell-mediated cytotoxicity after a respiratory infection. It has been demonstrated that factors such as diet can affect both the gut microbiota composition and that of the lung microbiota[146,147] . Recent studies on mice highlighted that dietary fibers and the short-chain fatty acids (SCFA) can protect against allergies and airways inflammations, modulating the immune system. The intake of fibers leads to an increase of SCFA, together with the modification of gut microbiota and, to a lesser extent, modification of the microbiota of the airways. Similar correlations have been made in humans between the modification of gut microbiota after the intake of fiber and a low incidence of asthma. A part of the microorganisms of the lung derives from the intestine. Firmicutes and Bacteroidetes prevail and characterize the gut microbiota[148]. The gut-lung microbiota relies fundamentally on the ability of the gut microbiota to modulate that of the lung and its immunologic activity (Figure A). The latter manifests it self through different elements: the production of substances such as lipopolysaccharides (LPS), the production of bacterial metabolites such as SCFA, the migration of immune cells (T-cells, in particular) from the gut to the lung through the blood. What is now evident from the literature is that a microbial dysbiosis of the lung can contribute to trigger the pulmonary diseases and to their progress, and this could be preceded by an intestinal dysbiosis [145]. The gut microbiome does not affect the intestine only, but it can affect very distant areas and alters the

immune response in other organs, such as the heart, the liver, and the kidney [145]. The same can likely happen in the lung[149].

The Microbial Dysbiosis in lung disease: chronic lung disorders and respiratory infection

Recent research has made it evident that a variety of chronic lung disorders, including asthma, COPD, and cystic fibrosis, are strongly linked to a dysbiotic airway microbiota[150,151,152,153-156]. This is usually the result of a loss in bacterial diversity[150,152,156] due to the outgrowth of certain pathogenic bacteria. The airway microbiota of patients with chronic lung disorders presents a disease-specific phenotype. In contrast to healthy individuals, those with asthma or COPD demonstrate an overrepresentation of Proteobacteria (in particular Haemophilus, Moraxella, and Neisseria spp.) and Firmicutes (Lactobacillus spp.), whereas the proportion of Bacteroidetes (specifically, Prevotella spp.) is significantly decreased[151,152]. The lung microbiota of patients with cystic fibrosis is characterized by a strong increase in typical cystic fibrosis pathogens of the Proteobacteria phylum, including Pseudomonas, Haemophilus, and Burkholderia, along with an additional outgrowth of the Actinobacteria phylum[157,158]. However, not only is the airway microbiota altered during these chronic lung disorders, but shifts in the composition of the intestinal microbiota have also been noted, particularly within the context of asthma and cystic fibrosis. A strong correlation has been made between low microbial diversity in the gut during early infancy and an asthmatic phenotype in childhood[163,164]. A study in mice has also shown that reducing the microbial load and diversity by antibiotic administration during the first 3 weeks of life exacerbates experimental allergic airway inflammation after adult exposure to aeroallergens[159]. This suggests a critical window early in life, during which microbial diversity in the intestine is important for appropriate systemic immune function later in life. During this early-life timeframe, a specific reduction in the prevalence of Bifidobacteria and an increase in Clostridia have been observed in the intestine of subjects with asthma[160]; however, whether this phenotype is transient or persistent has not been investigated. However, little is known about how viral and bacterial infections, the underlying causes of exacerbations of chronic lung disorders, can shape the microbiota. In a recent publication, Yi and colleagues[161] compared the bacterial microbiota of the upper respiratory tract of healthy individuals with patients acutely infected with influenza, parainfluenza, rhinovirus, respiratory syncytial virus, coronavirus, adenovirus, or metapneumovirus. Virus infected individuals generally demonstrated an increased prevalence of Haemophilus and Moraxella. However, no virus-specific bacterial profile

could be detected. Patients with chronic bacterial infections regularly present with either Pseudomonas aeruginosa or Haemophilus influenzae dominated disease. It has been shown that these two pathogens not only strongly compete with each other for the same habitat, but that infections with one or the other are also accompanied by a very distinct composition of the core microbiota[162]. Whereas Prevotella and Flavobacterium dominate in the P. aeruginosa–infected samples, Neisseria is significantly more abundant in the H. influenzae group [162]. These results indicate that bacterial and viral respiratory infections are accompanied by changes in the microbial composition, at least of the upper respiratory tract[165].

The Microbial dysbiosis in lung diseases associated with increased risk of infection caused by Aspergillus spp.

There is increasing evidence that the individual microbiome supervises the outcome of the host-fungus interaction by influencing mechanisms of immune regulation, inflammation, metabolism, and other physiological processes. Microbiome-mediated mechanisms of resistance allow therefore the control of fungal colonization, preventing the onset of overt disease, particularly in patients with underlying immune dysfunction. In this regard, it is reported the contribution of the microbiome-metabolome crosstalk to host immunity against A. fumigatus[166]. The function of the immune system is regulated by the microbiota and its metabolic activity, whereas the diversity of the microbiota and its commensal nature are kept under control by the immune system. The gut dysbiosis is often associated with the outgrowth of the yeast C. albicans that, in turn, leads to the development of allergic airway responses to A. fumigatus mediated by Th2 cells, M2-polarized macrophages capable of producing inflammatory mediators such as prostaglandin E2 (PGE2), and eosinophils. Segmented filamentous bacteria (SFB) are known to regulate Th17 immunity locally in the gut but also in the lung in response to A. fumigatus, and gut microbiota-derived metabolites such as short-chain fatty acids (SCFAs) have been identified as master regulators of pulmonary immune responses. In addition to these, lactobacilli are able to degrade tryptophan into immunoregulatory metabolites and induce the production of IL-22 to sustain immune tolerance to C. albicans. The microbial communities inhabiting the lung are, in turn, able to regulate gut immunity, for example by promoting the expansion of IFN-g-producing Th1 cells and recruitment to the gut, and inducing IgA class switch recombination and systemic humoral responses. Some of the bacterial species in the lung, most notably P. aeruginosa, interact directly (and indirectly via metabolites, including volatile organic compounds, VOCs) with A. fumigatus, contributing to the establishment of a permissive

environ- ment for fungal colonization, and under certain conditions, to overt disease. When dysbiosis occurs due to underlying pulmonary diseases, immune system dysfunction, or defects in the ciliary activity of the mucous pulmonary epithelium, fungal colonization in the lungs may become uncontrolled and exacerbate into overt fungal disease. The bacterial dysbiosis is associated with the outgrowth of certain bacterial communities at the expense of others (typically belonging to the Bacteroidetes phylum) and these altered profiles may be permissive for fungal colonization and disease. In particular, Patients with COPD and asthma share many resemblances in the bacteria causing dysbiosis, with an outgrowth of the Proteobacteria phylum (e.g., Haemophilus spp.) and a shift toward an increase in the pro- portion of streptococci and staphylococci within the Firmicutes phylum. Shifts in the bacterial communities in the CF lung are different, with an outgrowth of the Proteobacteria (which includes P. aeruginosa, B. cepacia and H. influenzae) and Actinobacteria phyla. An enrichment in the abundance of the Proteobacteria (particularly Pseudomonas spp.) and Firmicutes phyla is observed during H1N1 influenza infection[166].

AIM OF THE STUDY

The present project is aimed at understanding the possible role of pulmonary microbiota in pulmonary aspergillosis. Recent studies have shown that the lung of healthy non-smokers, mistakenly considered a sterile or near-district, is populated by different microbial communities. The composition of the microbiota of the upper and lower respiratory tract is not clear. Biopsies from different lung lobar regions have shown that the resident microbial population varies in the same subject. However, various studies have shown that in such diversity there is a "core" pulmonary microbiome common to all subjects.

The primary objective is the characterization of the lung microbiota in a cohorts of patients susceptible to fungal lung infections due to filamentous fungi, Aspergillus in particular, such as patients with malignant haematological diseases or those undergoing bone marrow transplantation. The project involves a prospective and observational study aimed at identifying the microbial and immunological factors responsible for susceptibility / resistance against pulmonary aspergillosis.

The secondary objective is the Identification of a microbial signature of the airway microbiome predictive of the risk of fungal infection.

Herein, the study of relationship of the airways dysbiosis and risk of IFI.

PATIENTS, MATERIALS AND METHODS

1- Study design and data collection

This was a non-profit, non-sponsored, multicentric and observational study. It was approved by the institutional review board both by the University of Perugia and by University of Parma and also by the local institutional review boards of SEIFEM group. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

The clinical part of the study was conducted at the Hematology and Bone Marrow Transplantation Unit of Parma. The laboratory part of the study (microbiota analysis) was conducted at Mycology laboratory of the SC of Microbiology AO of Perugia and the Department of Experimental Medicine of the University of Perugia.

One hundred seventy three patients diagnosed with hematological malignancies attending different hematological centers in Italy were enrolled between November 2015 and November 2017.

Inclusion criteria in patients with hematologic malignancies and invasive fungal infection:

- belonging to the Caucasian race,
- age >25 years,
- malignant haematological diseases and / or first hematopoietic stem cell transplantation,
- proven or probable invasive fungal infection developed after initiation of therapy and / or transplantation.

Inclusion criteria in control patients:

- belonging to the Caucasian race,
- age >25 years,
- malignant haematological diseases and / or first hematopoietic stem cell transplantation,
- documented absence of invasive fungal infection.

Information regarding age, gender, relation donor-patient gender, underlying disease and stage, transplant matching, cytomegalovirus serology of donors and patients, conditioning regimen, graft-versus-host disease and antifungal prophylaxis was obtained for all patients. Cases of invasive aspergillosis was identified from microbiologic and histopathological reports and classified as

"probable" or "proven" according to the revised standard criteria from the European Organization for Research and Treatment of Cancer/Mycology Study Group (EORTC/MSG).

Clinical records from each patient were reviewed and demographic and clinical data including age, gender, disease, chemotherapeutic regimen, neutropenia and antimicrobial treatments *(Table 5)* as well as transplantation type and conditioning regimens *(Table 6)* were showed.

2- Sample collection, processing and sequencing for microbial composition

The subjects enrolled, after having read and signed the informed consent, was subjected to the collection of nasal swabs (right and left) and pharyngeal in a single sampling session with a cadence of about 30 days. In particular, in patients undergoing bone marrow transplantation the swabs was taken before, during and up to six months after transplantation.
In total 945 pharyngeal swabs were collected, on average, monthly from the diagnosis and up to 6 months *(Table 5).* Swabbing was performed by the treating physicians at the time of outpatient visits according to standard procedures. Swabs were stored in Liquid Amies medium (Copan Diagnositics Inc.) in each participating center before delivery to the University of Perugia for sample extraction. On the day of extraction, pharyngeal samples were thawed on ice, transferred into 2 ml-eppendorf tubes, and centrifuged at 4°C for 15 min. The supernatant was collected and stored while the pellet further processed for DNA extraction and sequencing as described subsequently. In particular, the samples was processed to perform the following diagnostic investigation:
- Direct microscopic examination: it was evaluated the inflammatory status of the district and the microscopically detectable bacterial and / or fungal population
- Microbial profiling: it was assessed the microbial content in the samples by standard culture and molecular detection techniques.
- Metagenomics analysis: It was obtained the collection of genomes and genes from the members of a microbiota through gene amplification and sequencing of genes encoding for ribosomal RNA of bacterial and fungal species, cultivable and non-cultivable. The amplification of DNA and the sequencing of the hypervariable regions of the genes coding for ribosomal bacterial and fungal RNA was carried out at the Innovation Phase of Genomics Genetics and Biology of Perugia, following

validated protocols. The gene libraries was prepared using the Nextera XT DNA Sample Preparation Kit protocol, subsequently normalized and loaded onto the MiSeq platform (Illumina)

Following successful extraction, profiling was primarily based on polymerase chain reaction (PCR) amplification of the bacterial 16S rRNA gene (V3 and V4 variable regions) or the fungal-specific internal transcribed spacer (ITS) region on the Illumina MiSeq platform combined with in silico inference of metagenomic functions. A subsequent study of bacterial diversity in the airways based on RNA rather than DNA was carried out to profile active members of the airway microbiota. Reads was filtered and quality trimmed. Once spurious signal has been removed and profiles normalized, the identities of the microbes present within the samples was determined by comparing the sequencing obtained to a reference database. Quantitative real-time PCR (qPCR) employing primers specific for particular bacteria was also carried out molecular profiling of microbes.
- Metabolomic analysis: it was determined the metabolite profile(s) in any given strain or single tissue. Specifically, evaluation of the indole and kinurenine tryptophan metabolites. Human samples (first homogenized and then sonicated at ambient temperature (298 K) for 30 min to destroy bacteria) as well as culture broths from selected bacterial strains was subjected to targeted metabolomics at the Mass Spectrometry Centre (CISM) of the University of Firenze (Italy). Targeted metabolomics was performed by GC-EI MS on single and triple quadruple mass spectrometers (Agilent Technologies 5975 and Agilent Technologies 7000) and by HPLC-ESI (or APCI) HRMS on a LTQ Orbitrap (Thermo Fisher) and on a 6540 UHD Accurate Mass Q-TOF (Agilent Technologies), coupled to a HPLC Ultimate 3000 (Dionex) and a HPLC 1290 (Agilent Technologies), respectively. The high resolution mass spectrometers in conjunction with new performing interfaces for MS coupling to chromatographic techniques and potent bioinformatics tools was used for qualitative and quantitative analysis of targeted and untargeted metabolites. Data are visualized by means of principal component scores, where each point represents an individual metabolic/microbial profile.
- Genetic polymorphisms analysis performed by bidirectional PCR amplification of specific alleles (Bi-PASA). The Bi-PASA method uses a combination of four primers, two external primers and two internal allele-specific primers. The internal primers are characterized by a 5 'of 10-nucleotide rich in G + C that prevents "mega-priming" and aims the efficiency of amplification. A second of the genotype, Bi-PASA produces two or three superimposable fragments that give the identification of individuals heterozygous, homozygous wild-type and homozygous mutants for each polymorphism.

- 16S rRNA-based Microbiota Analysis. The fastq files was processed using QIIME (software Quantitative Insights Into Microbial Ecology) as previously described.

3- Statistical analysis

For human studies, demultiplexing of all libraries for each sequencing lane was accomplished by the Illumina bcl2fastq 2.17.1.14 software . Only reads with at least 100 nucleotides (nt) were retained and then primer sequences were detected, clipped and oriented into forward-reverse primer orientation. The forward and reverse paired-end reads were imported and analyzed by using Qiime2 platform[167] in a genomic cloud -computing environment based on[168,169] and oriented for biological nano-communication systems in blood vessels for early tumor medical diagnosis[169]. At first, paired-end sequences were denoised, dereplicated, filtered by both any phiX reads and chimera (consensus), by using q2-dada2 quality control method[170] for detecting and correcting (where possible) Illumina amplicon sequence data. In particular, the q2-dada2 method makes use of sequence error profiles to obtain putative error-free sequences, referred to as either sequence variants (SVs) or 100% operational taxonomic units (OTUs). It also truncates forward and reverse sequences at the first instance of a quality score less than or equal to 2. Reads with errors higher than 2 were discarded and only reads with a minimum overlap of 20 nt were retained and joined. SVs were assigned taxonomy using a Naive Bayes classifier model trained on the Silva138 99% database trimmed to the V4-V5 region of the 16S. The classifier was then applied to the obtained SVs for mapping them to taxonomy. A phylogenetic tree was constructed via sequence alignment with MAFFT [171], filtering the alignment and applying FastTree [172] to generate the tree. The analysis of the rarefaction curves of the Shannon index indicated a good sequencing quality as the richness index does not increase significantly with the sampling depth for each sample. In order to ease the comparison of microbial composition between groups, samples were normalized by rarefying sequencing to 104 reads. Moreover, in order to evaluate the composition of the microbial community of low abundant taxa accurately, a taxon was regarded as detected in a sample if it was counted at least 10 times in that sample, thus setting the resolution of composition analysis equal to 0.1%. Taxa with an abundance per sample lower than the given resolution were counted in the global community but not considered for differential analyses in order to avoid non-reliable comparisons of rare taxa between groups that could lead to biased analyses [173]. Adonis analysis based on distance matrix was performed to evaluate differences in microbial profiles (beta-

diversity) using non-parametric permutational multivariate analysis of variance (permanova and adonis) within QIIME2 with a P value of <0.05 through 9,999 permutations. The within sample alpha-diversity was assessed based on the 16S rRNA gene sequencing data, using Shannon and Chao1 diversity indexes estimated by using the QIIME2 platform. Corresponding statistical significances in sample groups comparison were determined using a Kruskal-Wallis test[174]. Also the between samples beta-diversity was assessed based on the 16S rRNA and estimates were calculated on the SVs within QIIME2 using Jaccard and Bray-Curtis distances between samples[175]. Principal coordinates were computed from the resulting distance matrices in order to separate quantitatively all sources contributing to the beta diversity. To reduce the dimensionality of the diversity investigation, the principal coordinate analysis (PCoA) was limited to the first three components, thus allowing visualization of the most effective relationships contributing to diversity between groups of samples. Permutational MANOVA[176] with 999 permutations was used to test significant differences between sample groups based on both Jaccard and Bray-Curtis distance matrices. 16S SVs were agglomerated into Phylum, Class, Order, Family, Genus and Species levels within QIIME2 for evaluating the corresponding taxonomic abundance. The LEfSe (Linear discriminant analysis effect size) [177] was used to test the association at each taxonomic level. LEfSe employs a non-parametric Kruskal-Wallis sum-rank test to differentiate between class features and a subsequent LDA to estimate effect size of taxa which violated the null hypothesis. LEfSe generates its initial model using the interaction 'y ~ x' where y is the taxon abundance and x is the class variable of interest. Only after examining the null hypothesis, the LDA model is built and used to calculate effect sizes based on biological class. LEFSE has been applied with default alpha values for the Anova and Wilcoxon test (0.05) and the LDA effect size has been evaluated by setting the absolute value of the logarithmic LDA threshold equal to 3.5. Other LEFSE parameters have been set to the default. LEfSe outcomes were also reported on heat trees, i.e. visualization based on a community taxonomic heat maps-used to depict statistics for every taxon[178,179]. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) pipeline was applied to predict metagenome functions from 16S metagenomic samples. In particular, the enzyme-catalyzed reaction (EC number), functional gene content based on KEGG database annotations for reference genomes (KEGG Orthology), and metabolic pathway abundances of pharynx microbial communities using the pathway rules from MetCyc database were predicted with PICRUSt2[180,181]. Sequenced samples were provided as ASV abundance tables (rarefied at 3000 reads) and files with representative sequences. To estimate the extent of change in microbial metabolic pathway representation across High and

Low risk, LEfSe was applied to PICRUSt2 outcome in order to identify a number of metabolic pathways that were significantly differentially represented in the predicted metagenomes. Box plots of predicted abundances were produced in R using ggplot2 package. In order to compare L-kyn, L-trp and 3-IAld metabolomic concentrations between groups, data were log-transformed after normalization with the Pareto transform [182], and the Grubb's test recursively applied in order to test for outliers. Then, a non-parametric Mann-Whitney test was used for evaluating significant differences between groups.

RESULTS

1- The oropharyngeal microbiota of hematologic patients

In a first analysis of the SNIF database, we focused on the oropharyngeal microbiome owing to its higher richness, that may increase the likeliness of uncovering associations with the risk of IFI, for being the main source of the lung microbiota in adults tract[183] and for the potential protective role in pulmonary infections[184] The taxonomic composition of the oropharyngeal microbiome is shown in *Figure 1.* Consistently with previous reports[185,135] Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria and Fusobacteria represented the major phyla accounting for nearly the entire spectrum of bacteria detected in the oropharynx, followed by low abundant phyla with a cumulative abundance of less than 1% (*Figure 1A)*. At the genus level, Streptococcus was found to be the most abundant, followed by Veillonella, Prevotella, Neisseria, Actinomyces, Haemophilus, Fusobacterium, and Rothia, common genera of the pharynx[183,185,135] (*Figure 1B*).

2- Hematologic patients at different risk for IFI harbor distinct oropharyngeal microbiomes

To define the potential predictive value of the oropharyngeal microbiome, each sample was associated with the overall risk of IFI, based on the following criteria, i.e., the duration of neutropenia, antibiotic usage and the dynamic risk proposed by SEIFEM (Epidemiology Survey of Invasive Fungal Infections in Hematological Malignancies) [3] . The details for risk stratification in each hematological malignancy, based on diagnosis, phase and type of treatment, as well as the algorithm at the basis of the dynamic risk definition are described previously. Upon application of these criteria, samples were assigned to high risk (HR) for IFI if collected during a condition of prolonged neutropenia or administration of broad-spectrum antibiotics, or if satisfying the conditions for dynamic high-risk (dHR) according to SEIFEM algorithm. Conversely, samples were assigned to low risk (LR) for IFI if collected outside periods of prolonged neutropenia or broad-spectrum antibiotics administration or if satisfying the conditions for dynamic low-risk (dLR) according to SEIFEM algorithm. The distribution for age, gender and hematological disease for the HR and LR populations considered separately are shown in *Supplementary Figure 1.* The overlap between the different HR and LR populations is shown in *Supplementary Figure 2.* Specifically, 66.4% and 94% of the LR samples are identified by all the three criteria or at least two of them, respectively,

indicating a substantial degree of overlapping in the classification of patients as low risk *for IFI (Supplementary Figure 2A*). Conversely, only 15.4% and 29.6% of HR samples are identified by all the three criteria or at least two of them, respectively (*Supplementary Figure 2B-E).* The difference is driven by a group of samples that are recognized as HR by the dynamic risk proposed by SEIFEM, but not by prolonged neutropenia and administration of broad-spectrum antibiotics. This is in line with the dynamic risk encompasses risk factors that go beyond the occurrence of neutropenia and the use of broad-spectrum antibiotics (3). The analysis of alpha and beta diversities revealed that LR and HR samples were associated with distinct microbiota for all the criteria. Indeed, a higher richness and evenness, as measured by observed OTUs, Chao1 and Shannon indexes, were observed in the LR groups, i.e., no prolonged neutropenia or broad-spectrum antibiotics or assigned to dLR, than the HR groups (*Figure 2A*). This result was rather expected as the HR group is associated with a long period of neutropenia and therefore more intense pharmacological treatment that severely affects the microbial composition and diversity. The oropharyngeal microbiota of the two groups also showed significant differences in compositional structure, as measured by Jaccard and Bray-Curtis indexes (*Figure 2B*). Indeed, PCoA analysis derived from Jaccard and Bray-Curtis distances revealed significative differences between the LR and HR groups *(Supplementary Figure 3).* Upon applying the Adonis test to Jaccard and Bray-Curtis diversities when a linear simple multifactorial model of the dynamic risk, prolonged neutropenia, broad-spectrum antibiotics and occurrence of mucositis was used, all the variables, except mucositis, had significant different profile for both metrics. Of note, the dynamic risk accounted for the largest percentage of variation (*Table 7*). Collectively, these results indicate that the different criteria provide a consistent framework to identify potential signatures of the oropharyngeal microbiome that could predict the risk of IFI.

3- The risk of IFI is associated with distinct microbial genera

In order to identify these signatures, we performed high dimensional class comparisons using linear discriminant analysis of effect size (LEfSe) [186] by which we could detect significant differences in the bacterial communities between LR and HR groups. Despite differences at the level of some genera, a common signature could be identified that was associated with the risk of infection among the differently stratified groups. Specifically, we observed the relative predominance of members of Bacteroidetes (i.e., the Prevotella genus), Firmicutes (i.e., Clostridia with the Lachnospiranaceae family) and Actinobacteria phyla, in addition to a higher abundance of most oral taxa (Veillonella, Neisseria, Leptotrichia and Gemella), in the LR groups. Conversely, the relative predominance of

members of Firmicutes (i.e. the Staphylococcus and Enterococcus genus) variably associated with Lactobacillaceae and Gram-negative Proteobacteria (i.e, Acinetobacter and Stenotrophomonas genus) was observed in the different HR groups (*Figures 3-5 and Supplementary Figures 4-5).* Similar results were obtained when samples were assigned to HR or LR for IFI if collected or not during the occurrence of mucositis (*Supplementary Figure 6*). The relative abundance of these genera are shown in *Supplementary Figure 4.*

4- The risk of IFI is associated with distinct microbial metabolic activities

To improve the predictive value of microbial signatures[187] , we have assessed microbial functional activity in dHR and dLR groups. By using PICRUSt2 and the annotated MetaCyc database, abundance of functional pathways were inferred from 16S data and LEfSe was applied in order to evaluate significant association to either HR or LR samples. In particular, genes involved in the biosynthesis of trp, glycolysis and homolactic fermentation were more abundant in HR than LR patients, while genes involved in the fatty acid elongation and the starch degradation pathways abundantly present in oral bacteria [188] were more abundant in LR than HR patients *(Figure 6A and Supplementary Figure 7).* The KEGG database confirmed the association of trp biosynthesis with the HR group *(Figure 6B and Supplementary Figure 8).* The increased trp auxotrophy would also predict different availability of trp in the HR and LR groups. We measured these levels in a subgroup of pharyngeal samples from 14 consecutive patients undergone HSCT during the study period and found that trp levels were indeed lower in HR than LR patients *(Figure 6C)*. Moreover, and importantly, while the levels of kyn, resulting from host catabolism of trp, were not different between the two groups, the production of indole-3-aldehyde (3-IAld), an indole that reflects the microbial consumption of trp, was significantly lower in HR than LR patients *(Figure 6C).* These findings suggest an apparent restricted availability of trp for microbial consumption in HR patients. Consistently, amino acid biosynthetic pathways were abundantly present in HR samples *(Supplementary Figure 7).* Collectively, these results reveal the existence of a transcriptionally active oropharyngeal microbiota that may impact on lung immune status and suggest that not only microbial composition but also active functional activity characterizes the pharyngeal microbiota.

DISCUSSION AND CONCLUSION

Herein, we have described the potential role of oropharyngeal microbiota in predicting the risk of IFI in hematologic patients. We have found that loss of alpha diversity, associated with decreased abundance of Clostridiales, Bacteroidetes, and common oral taxa, the relative expansion of Staphylococcus and Enterococcus, and a distinct metabolic profile characterize the HR patients. Interestingly, depletion of common taxa typically associated with oral health, including Actinomyces, Gemella and Veillonella, has been reported in oral microbiota of patients undergoing 5-fluorouracil or doxorubicin-based chemotherapy, and negatively correlated with mucositis severity[189]. Conversely, the role of Staphylococcus species in chronic inflammatory airway diseases[190] and infections in various hematological malignancies[191] is well documented. Interestingly, the expansion of Staphylococcus in oral and stool microbiome has been reported in a leukemic patient prior to invasive mucormycosis[192]. The increased prevalence of the Enterococcus genus in HR patients is also of particular interest, as it is not only present in chronic obstructive pulmonary disease[193] but also in the pharyngeal microbiome of patients with hematological malignancies[194] and is causally associated with GvHD in acute myeloid leukemia (AML) patients[195]. In addition, in a mouse intravenous chemotherapy model upon 5-fluorouracil administration, C. albicans infection resulted in increased abundance of Enterococcus genus in the oral mucosa, potentially responsible for the mucosal barrier impairment and fungal invasion[196]. The metabolic profiling helped to elucidate the possible pathogenic role of the HR associated microbes to dysbiosis. While the glycolytic pathways are generally associated with inflammation, the increased biosynthesis of trp is of particular interest as bacterial trp prototrophy has been associated with immune evasion and adaptation[197,198]. Indeed, on a metabolic level, the increased trp biosynthesis was associated with low levels of trp and indole observed in HR patients. Considering the influence of host and microbial trp metabolism on human health status[199,200] as well as the occurrence of metabolic immune programming during pulmonary infections[201], these results point to a dysregulated metabolic activity linked to bacterial virulence and host inflammation[200]. Fecal indole, as potential surrogate markers for microbial diversity and specific taxa, have been recently described in HSCT recipients[201]. As antibiotics alter the metabolic state of bacteria[203], it is likely that the more intense prophylactic regiment in HR patients is responsible for the observed metabolic profile in addition to the loss of bacterial diversity. In this regard, although specific Prevotella species

may exhibit different properties[204], the general beneficial role of Bacteriodetes in lung homeostasis[204], immune tolerance[205] and prevention of Th17 cell activation[206] is consistent with the abundance of Prevotella spp in LR patients and the loss in HR patients. Similar to what observed in patients with AML following induction therapy[207], in our HR cohort, we did not observe the expansion of pathogenic Proteobacteria probably because of first-line therapy with broad-spectrum piperacillin/tazobactam or carbapenems. Interestingly, however, Gammaproteobacteria that usually benefit from airway inflammation[208] and whose domination in the feces was a strong independent predictor of pulmonary complications in HSCT[217], were more abundantly present in HR than LR patients. Both Acinetobacter baumannii and Stenotrophomonas maltophilia are fatal infectious complications in HSCT[209,210]. Overall, these results, while consistent with previous findings in gut[217], provide evidence for significant differences in oral microbial composition of patients at different risk for IFI. Supported by the high number of patients enrolled, the results clearly highlight the major contribution of neutropenia, the associated antibiotic usage and the occurrence of mucositis in the differences observed between HR and LR patients. As such, these results may pay the way for further studies to uncover associations between the many different risk factors for IFI and the changes in the microbiome. In addition, considering the tolerability and rapidity of the oropharyngeal swabbing, including the advantage of self-collection[211], this sampling method appears highly feasible and relatively at low cost. Thus, if corroborated by further studies, the loss of alpha diversity associated with the loss of beneficial Clostridiales and Bacteroidetes could help delineate patients at risk of IFI, thereby providing information for antimicrobial therapy optimization. Indeed, HR patients would benefit from an antifungal prophylactic-based approach, as opposed to LR patients, for whom a fungal diagnostic-based approach is recommended to reduce overtreatment and unintended collateral damage to beneficial commensals. Discriminating patients who will benefit or not from antimicrobial prophylaxis will also help reducing the antimicrobial resistance crisis. In this regard, this study is a proof-of-concept demonstration that microbial metabolites, such as 3-IAld, known to protect from GvHD in murine HSCT[212], may also reduce the risk of IFI in these patients. Indeed, 3-IAld could protect mice against aspergillosis likely by activating AhR in ILC3 and inducing the production of IL-22. This mechanism has been recently shown to protect mice against mucosal candidiasis and dextran sodium sulfate-induced colitis [216,213]. Our results would expand the potential application of 3-IAld in the respiratory tract, in line with its suggested efficacy in cystic fibrosis[214]. These wide-ranging activities are associated with a favorable safety profile[215], as expected for a molecule endogenously produced in physiological conditions, a

fundamental prerequisite for clinical translation. More generally, this type of study reinforces the notion that mining the microbiota for microbial and metabolite-based therapies could instruct for future interventional strategies in patients at risk for IFI.

FIGURES AND TABLES

Fungus	Microscopic Analysis: Sterile Material	Culture: Sterile Material	Blood	Serology	Tissue Nucleic Acid Diagnosis
Molds[a]	Histopathologic, cytopathologic, or direct microscopic examination[b] of a specimen obtained by needle aspiration or biopsy in which hyphae or melanized yeast-like forms are seen accompanied by evidence of associated tissue damage	Recovery of a hyaline or pigmented mold by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding BAL fluid, a paranasal or mastoid sinus cavity specimen, and urine	Blood culture that yields a mold[c] (eg, *Fusarium* species) in the context of a compatible infectious disease process	Not applicable	Amplification of fungal DNA by PCR combined with DNA sequencing when molds are seen in formalin-fixed paraffin-embedded tissue
Yeasts[a]	Histopathologic, cytopathologic, or direct microscopic examination of a specimen obtained by needle aspiration or biopsy from a normally sterile site (other than mucous membranes) showing yeast cells, for example, *Cryptococcus* species indicating encapsulated budding yeasts or *Candida* species showing pseudohyphae or true hyphae[d]	Recovery of a yeast by culture of a sample obtained by a sterile procedure (including a freshly placed [<24 hours ago] drain) from a normally sterile site showing a clinical or radiological abnormality consistent with an infectious disease process	Blood culture that yields yeast (eg, *Cryptococcus* or *Candida* species) or yeast-like fungi (eg, *Trichosporon* species)	Cryptococcal antigen in cerebrospinal fluid or blood confirms cryptococcosis	Amplification of fungal DNA by PCR combined with DNA sequencing when yeasts are seen in formalin-fixed paraffin-embedded tissue
Pneumocystis	Detection of the organism microscopically in tissue, BAL fluid, expectorated sputum using conventional or immunofluorescence staining	Not applicable	Not applicable	Not applicable	Not applicable
Endemic mycoses	Histopathology or direct microscopy of specimens obtained from an affected site showing the distinctive form of the fungus	Recovery by culture of the fungus from specimens from an affected site	Blood culture that yields the fungus	Not applicable	Not applicable

Abbreviations: BAL, bronchoalveolar lavage; PCR, polymerase chain reaction.

[a]If culture is available, append the identification at the genus or species level from the culture results.

[b]Tissue and cells submitted for histopathologic or cytopathologic studies should be stained using Grocott-Gomori methenamine silver stain or periodic acid Schiff stain to facilitate inspection of fungal structures. Whenever possible, wet mounts of specimens from foci related to invasive fungal disease should be stained with a fluorescent dye (eg, calcofluor or blankophor).

[c]Recovery of *Aspergillus* species from blood cultures rarely indicates endovascular disease and almost always represents contamination.

[d]*Trichosporon* and yeast-like *Geotrichum* species and *Blastoschizomyces capitatus* may also form pseudohyphae or true hyphae.

***TABLE 1**: Criteria for Proven Invasive Fungal Disease (from Donnelly et al, CID 2021)*

Host factors

- Recent history of neutropenia ($<0.5 \times 10^9$ neutrophils/L [<500 neutrophils/mm^3] for >10 days) temporally related to the onset of invasive fungal disease
- Hematologic malignancy[a]
- Receipt of an allogeneic stem cell transplant
- Receipt of a solid organ transplant
- Prolonged use of corticosteroids (excluding among patients with allergic bronchopulmonary aspergillosis) at a therapeutic dose of ≥0.3 mg/kg corticosteroids for ≥3 weeks in the past 60 days
- Treatment with other recognized T-cell immunosuppressants, such as calcineurin inhibitors, tumor necrosis factor-α blockers, lymphocyte-specific monoclonal antibodies, immunosuppressive nucleoside analogues during the past 90 days
- Treatment with recognized B-cell immunosuppressants, such as Bruton's tyrosine kinase inhibitors, eg, ibrutinib
- Inherited severe immunodeficiency (such as chronic granulomatous disease, STAT 3 deficiency, or severe combined immunodeficiency)
- Acute graft-versus-host disease grade III or IV involving the gut, lungs, or liver that is refractory to first-line treatment with steroids

Clinical features

Pulmonary aspergillosis

The presence of 1 of the following 4 patterns on CT:

- Dense, well-circumscribed lesions(s) with or without a halo sign
- Air crescent sign
- Cavity
- Wedge-shaped and segmental or lobar consolidation

Other pulmonary mold diseases

As for pulmonary aspergillosis but also including a reverse halo sign

Tracheobronchitis

- Tracheobronchial ulceration, nodule, pseudomembrane, plaque, or eschar seen on bronchoscopic analysis

Sino-nasal diseases

- Acute localized pain (including pain radiating to the eye)
- Nasal ulcer with black eschar
- Extension from the paranasal sinus across bony barriers, including into the orbit

Central nervous system infection

1 of the following 2 signs:

- Focal lesions on imaging
- Meningeal enhancement on magnetic resonance imaging or CT

Mycological evidence

- Any mold, for example, *Aspergillus*, *Fusarium*, *Scedosporium* species or Mucorales recovered by culture from sputum, BAL, bronchial brush, or aspirate
- Microscopical detection of fungal elements in sputum, BAL, bronchial brush, or aspirate indicating a mold

Tracheobronchitis

- *Aspergillus* recovered by culture of BAL or bronchial brush
- Microscopic detection of fungal elements in BAL or bronchial brush indicating a mold

Sino-nasal diseases

- Mold recovered by culture of sinus aspirate samples
- Microscopic detection of fungal elements in sinus aspirate samples indicating a mold

Aspergillosis only

Galactomannan antigen

Antigen detected in plasma, serum, BAL, or CSF

Any 1 of the following:

- Single serum or plasma: ≥1.0
- BAL fluid: ≥1.0
- Single serum or plasma: ≥0.7 and BAL fluid ≥0.8
- CSF: ≥1.0

Aspergillus PCR

Any 1 of the following:

- Plasma, serum, or whole blood 2 or more consecutive PCR tests positive
- BAL fluid 2 or more duplicate PCR tests positive
- At least 1 PCR test positive in plasma, serum, or whole blood and 1 PCR test positive in BAL fluid

Aspergillus species recovered by culture from sputum, BAL, bronchial brush, or aspirate

Probable invasive fungal diseases (IFD) requires the presence of at least 1 host factor, a clinical feature and mycologic evidence and is proposed for immunocompromised patients only, whereas proven IFD can apply to any patient, regardless of whether the patient is immunocompromised. Probable IFD requires the presence of a host factor, a clinical feature, and mycologic evidence. Cases that meet the criteria for a host factor and a clinical feature but for which mycological evidence has not been found are considered possible IFD. (1,3)-beta-D glucan was not considered to provide mycological evidence of any invasive mold disease.

Abbreviations: BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; CT, computed tomography; PCR, polymerase chain reaction.

[a]Hematologic malignancy refers to active malignancy, in receipt of treatment for this malignancy, and those in remission in the recent past. These patients would comprise largely acute leukemias and lymphomas, as well as multiple myeloma, whereas patients with aplastic anemia represent a more heterogeneous group of individuals and are not included.

***TABLE 2**: Criteria for Probable Invasive Mold Disease (from Donnelly et al, CID 2021)*

Leukemia related	Host related	Treatment related factors	Fungal exposure
Lower Probability of CR (Adverse Cytogenetic/gene mutation profiles; WBC > 50.000/μL; Secondary AML	Age >65 yrs	Expected treatment related severe and prolonged neutropenia (ANC <100/μL for >10 d)	Rooms without HEPA filtration; Building constructions or renovations/ recent house renovation
Baseline neutropenia with ANC < 500/μL for > 7 d; MDS-related phagocytic dysfunction.	Organ dysfunction with High comorbidity index or Poor Performance status (≥2)	Highly mucotoxic regimen	Documented Airway Colonization By Aspergillus species
Leukemia status: relapse-refractory > first induction > consolidation	Chronic obstructive pulmunary disease. Active smoking	Mucositis grade ≥3 for >7 days, especially if involving lower gut.	Prior Aspergillosis
Persistance of day 15 bone marrow blast cells	Immunity polymorphism		Multisite colonization by Candida species.
No CR by end of induction phase	Pharmacogenomics of antineoplastic drugs		Jobs with high exposure (farming, gardening, contruction work)

CR = Complete Remission; ANC = absolute Neutrophils count; WBC = White Blood cells.

TABLE 3: *Risk factors for IFIs in AML (from Pagano et al, Blood Rev 2016)*

Risk stratification of HMs for diagnosis, phase and kind of treatment.

High risk	Intermediate risk	Low risk
AML undergoing Induction CHT with any of the following Risk Factors: Neutropenia at baseline, low CR probability (Adverse K, secondary AML), age >65 yrs., significant pulmonary disfunction, high e-TRM score.	AML not meeting criteria for High or Low Risk groups.	AML <45 yrs.; Undergoing first remission-induction or consolidation CHT and without ANY Risk Factors for IFI
AML with Prior IA		APL treated with ATRA/ATO
AML undergoing salvage regimens for Relapsed/Refractory disease.		
Allogeneic Stem Cell transplantation (from donors other than a matched sibling donor, patients active HM, GVHD requiring high-dose steroids and history of previous IFI)	Allogeneic Stem Cell transplantation (from matched sibling donors, patients in complete remission with no evidence of GVHD and no previous IFI)	
MDS/AML receiving azacitidine as salvage therapy after intensive regimens	MDS with IPSS >1.5 treated with azacitidine 75 mg/m(2) for 7 days MDS during the first 2–3 cycles of AZA/Decitabine	
Acute lymphoblastic leukemia: elderly patients (≥55 y); intensive pediatric regimens (induction);HD dexametazone; previously treated (relapsed/refractory)	Acute Lymphoblastic Leukemia: Adults (30–54 y); Standard induction chemotherapy; Intensive consolidation treatment; TKI + reduced cht (Ph + ALL)	Acute Lymphoblastic Leukemia: Younger adults (30 y); Maintainance treatment (complete remission); TKI + steroids (Ph + ALL)
	Autologous Stem Cell Transplantation: Previous IFI; >3 lines of therapy (disease burden); Prolonged neutropenia (ANC <500/mm3 for more than 14 days); corticosteroid therapy; Colonization by *Candida* spp.; Previous Fludarabine treatment	MPN (Chronic Myeloid Leukemia, Essential Thrombocitemia, Idiopathic Thrombocytosis, Policytemia Vera)
	CLL treated with multiple lines of CTX Multiple myeloma in 3 or more lines or during ASCT DLBCL relapsed/refractory HD if treated with "escalating BEACOPP"	Low or high grade NHL, CLL, MM, HD treated with conventional frontline chemotherapy

AML: acute myeloid leukemia APL: acute promyelocytic leukemia MDS/AML: myelodisplastic sindromes/ acute myeloid leukemia MPN: myeloproliperative neoplasms CLL: chronic lymphocytic leukemia MM: multiple myeloma HD: Hodgkin's disease NHL: non-Hodgkin's lymphoma.

TABLE 4: *Risk Stratification of HM (from Pagano et al, Blood Rev 2016)*

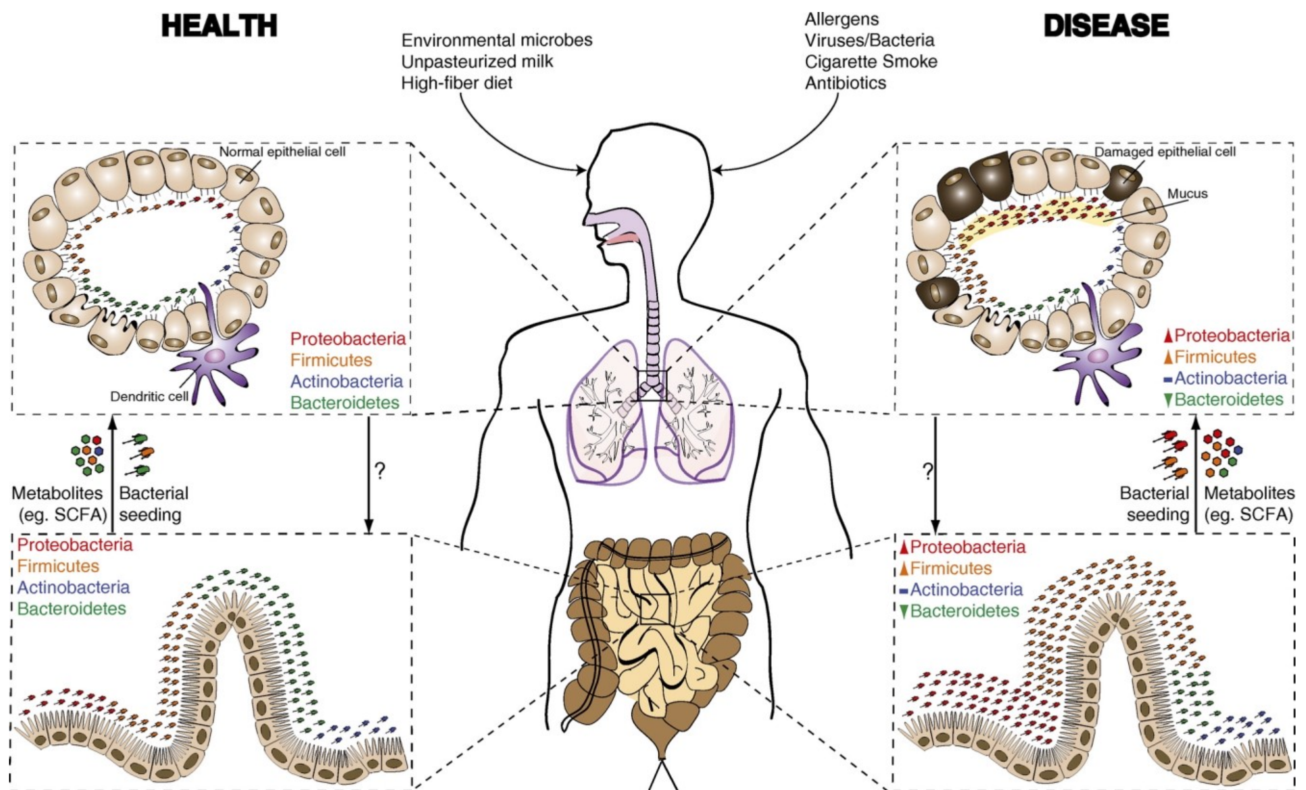


Figure A. ***Intestinal–pulmonary cross-talk during respiratory health and disease.*** *In healthy individuals, the intestinal and airway microbiotas harbor diverse communities, which are predominated by the phyla, Bacteroidetes and Firmicutes. During respiratory disease a dysbiosis of both the intestinal and airway microbiota has been reported, commonly presenting as an outgrowth of Proteobacteria and Firmicutes. A cross-talk between these two microbiota compartments has been proposed. The intestinal microbiota influences pulmonary microbial composition and immune responses by both direct seeding of the respiratory tract with bacteria and the distribution of bacterial metabolites, such as short-chain fatty acids (SCFAs), which promote the growth of certain SCFA-producing bacteria (e.g., Bacteroidetes) and/or act directly as immunomodulatory molecules (from Marsland, Trompette, and Gollwitzer: Gut–Lung Axis in Respiratory Disease, Ann Am Thorac Soc Vol 12, Supplement 2, pp S150–S156, Nov 2015) .*

Number of patients total	*173*
Age in years, median (range)	*54,5 (range 25-65)*
Female n (%)	*72 (41,6)*
Male n (%)	*101 (58,4)*
SAMPLE n (%)	
Total	*2830*
Nasal swab	*1885(66,6)*
Pharyngeal swab	*945 (33,4)*
UNDERLYING DISEASE n (%)	
Acute Myeloid Leukemia	*51(29,5)*
Acute Lymphoid Leukemia	*9(5,2)*
Lymphoma	*58(33,5)*
Myeloma	*44(25,5)*
Chronic Lymphatic Leukemia	*3 (1,7)*
Mielofibrosis	*2(1,1)*
Chronic Myeloid Leukemia	*1(0,6)*
Aplastic Anemia	*1 (0,6)*
Others	*4 (2,3)*
DISEASE STATUS n (%)	
At diagnosis	*59 (34,1)*
Complete Remission	*63(36,4)*
Partial Remission	*19 (11)*
Relapsed / Refractory	*14(8,1)*
Progression	*18 (10,4)*
TREATMENT n (%)	
Total cycle administred	*424*
Myeloablative chemotherapy	*201 (47,4)*
Reduced intensity chemotherapy	*67(15,8)*

Others (i.e. monoclonal antibodies, proteasome inhibitors and immunomodulatory drugs)	*156 (36,8)*
STEROID THERAPY during treatment , n (%)	
Number of cycle	*295(69,6)*
Duration > 7 days	*94(31,9)*
NEUTROPENIA during treatment, n (%)	
Severity (<500 neutrophils/mcl)	*213 (50,2)*
Duration > 10 days	*135 (63,4)*
ANTIBIOTICS, n (%)	
Number of cycle	*458*
*Prophylaxis **	*105 (22,9)*
Therapy	*353 (77,1)*
Broad-spectrum antibiotics	*258 (73)*
(i.e. piperacillin-tazobactam or ceftazidime +/- amynoglycoside or tigecycline; carbapenem +/- aminoglycoside or tigecycline)	
Narrow-spectrum antibiotics	*95 (27)*
(i.e. fluorochinolones or oral penicillin but not antimicrobial combinations)	
** 73 of 105 (69,5) are PCP prophylaxis and 32 of 105 (30,5) are antibacterial prophylaxis*	
ANTIFUNGALS, n (%)	
Number of cycle	*159*
Prophylaxis	*105 (66)*
Therapy	*54 (34)*

TABLE 5*: Characteristics of patients*

Number of patients total	*70*
Age in years, median (range)	*54 (range 25-65)*
Female n (%)	*35 (50)*
Male n (%)	*35 (50)*
TYPE OF HSCT, n (%)	
Autologous	*37 (53)*
Allogeneic	*33 (47)*
Matched Related Donor	*9 (27,3)*
Matched Unrelated Donor	*3 (9,1)*
Mismatched Related Donor	*15 (45,4)*
Mismatched Unrelated Donor	*6 (18,2)*
STEM CELL SOURCE, n (%)	
Peripheral Blood	*61 (87,1)*
Bone marrow	*9 (12,9)*
UNDERLYING DISEASE, n (%)	
Acute Myeloid Leukemia	*18 (25,7)*
Acute Lymphoid Leukemia	*6 (8,6)*
Lymphoma	*21 (30)*
Myeloma	*23 (32,85)*
Others	*2 (2,85)*
DISEASE STATUS AT TRANSPLANT, n (%)	
Complete Remission	*55 (78,6)*
Partial Remission	*8 (11,4)*
Relapsed / Refractory	*2 (2,9)*
Progression	*5 (7,1)*
CONDITIONING REGIMEN, n (%)	
Reduced Intensity	*4 (5,7)*
Myeloablative	*66 (94,3)*
CONDITIONING REGIMEN FOR ALLOGENEIC TRANSPLANT, n (%)	
TBI-based	*2 (6,1)*
Chemotherapy-based	*31 (93,9)*
DONOR/RECIPIENT CMV SEROLOGY, n (%) *	
Neg/Neg	*0 (0)*
Neg/Pos	*9 (27,3)*
Pos/Neg	*2 (6,1)*
Pos/Pos	*19 (57,6)*
** serology not note in 3 patients*	

TABLE 6: *Characteristics of patients undergone hematopoietic stem cell transplantation*

Jaccard

Parameter	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Dynamic risk	1	2.361346588	2.361346588	7.203580256	0.024817729	0.001
Prolonged neutropenia	1	0.721783724	0.721783724	2.201890654	0.00758594	0.001
Broad-spectrum antibiotics	1	0.594637061	0.594637061	1.81401401	0.00624963	0.001
Mucositis	1	0.340901612	0.340901612	1.039962593	0.003582873	0.33
Residuals	278	91.12890092	0.327801802	NA	0.95776383	NA
Total	282	95.1475699	NA	NA	1	NA

Bray-Curtis

Parameter	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Dynamic risk	1	1.579643048	1.579643048	5.350979996	0.018505499	0.001
Prolonged neutropenia	1	0.839875287	0.839875287	2.845045193	0.009839129	0.001
Broad-spectrum antibiotics	1	0.608823486	0.608823486	2.062366115	0.00713236	0.003
Mucositis	1	0.265042627	0.265042627	0.897821693	0.003104971	0.614
Residuals	278	82.06735358	0.295206308	NA	0.961418042	NA
Total	282	85.36073802	NA	NA	1	NA

TABLE 7: *Multivariate analysis*

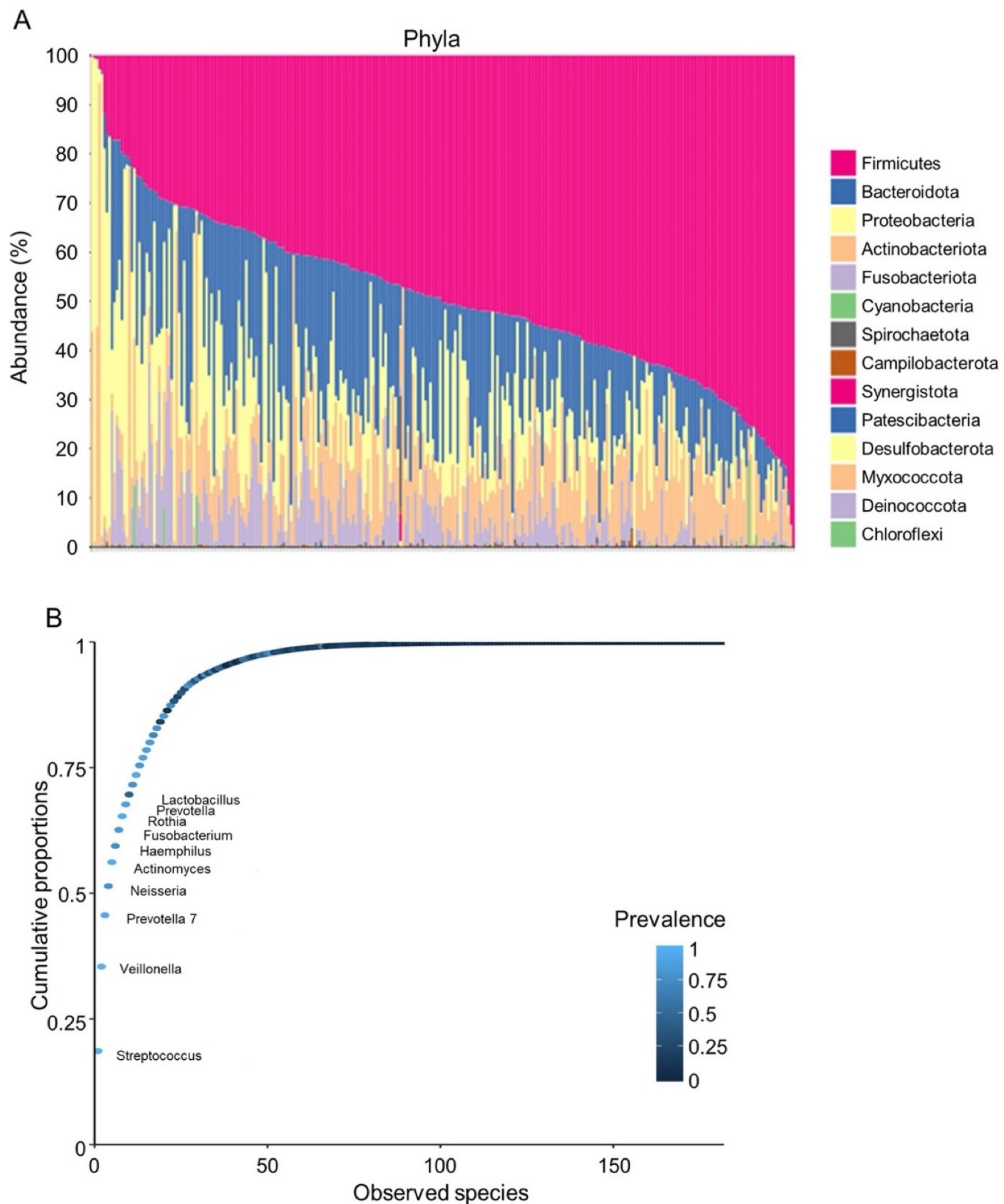


Figure 1**. The oropharyngeal microbiome of hematologic patients is dominated by bacteria commonly associated with the oropharynx.* ***(A) *Barplot showing bacterial composition (abundance percentage) of each sample at phylum level. Taxa are differentiated by colors. Samples are ranked based on the abundance of Firmicutes.* ***(B)*** *Behavior of the cumulative proportions of taxa at genus level in the complete cohort versus number of observed taxa. Color of each taxon represents the prevalence of the taxon within the community. Taxa are ordered by increasing percentage value and the first 10 most abundant genera are explicitly indicated by labels. These 10 taxa present a prevalence >50%.*

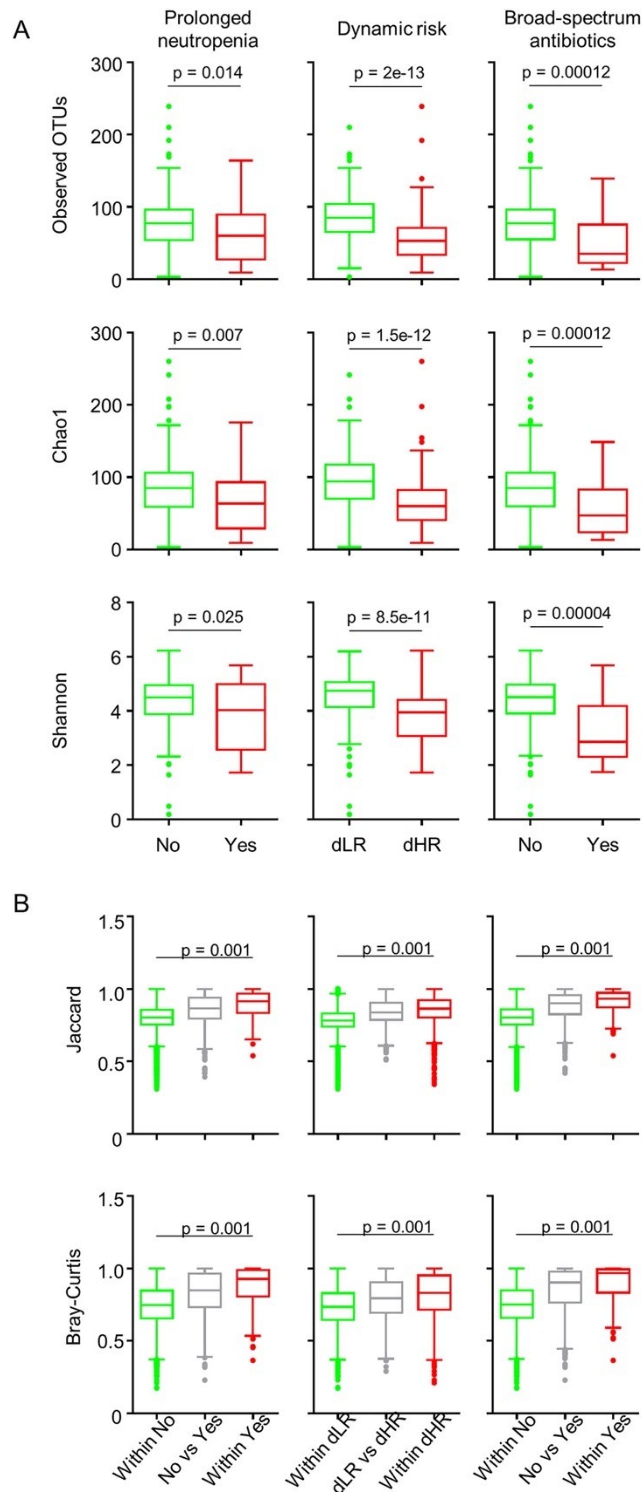
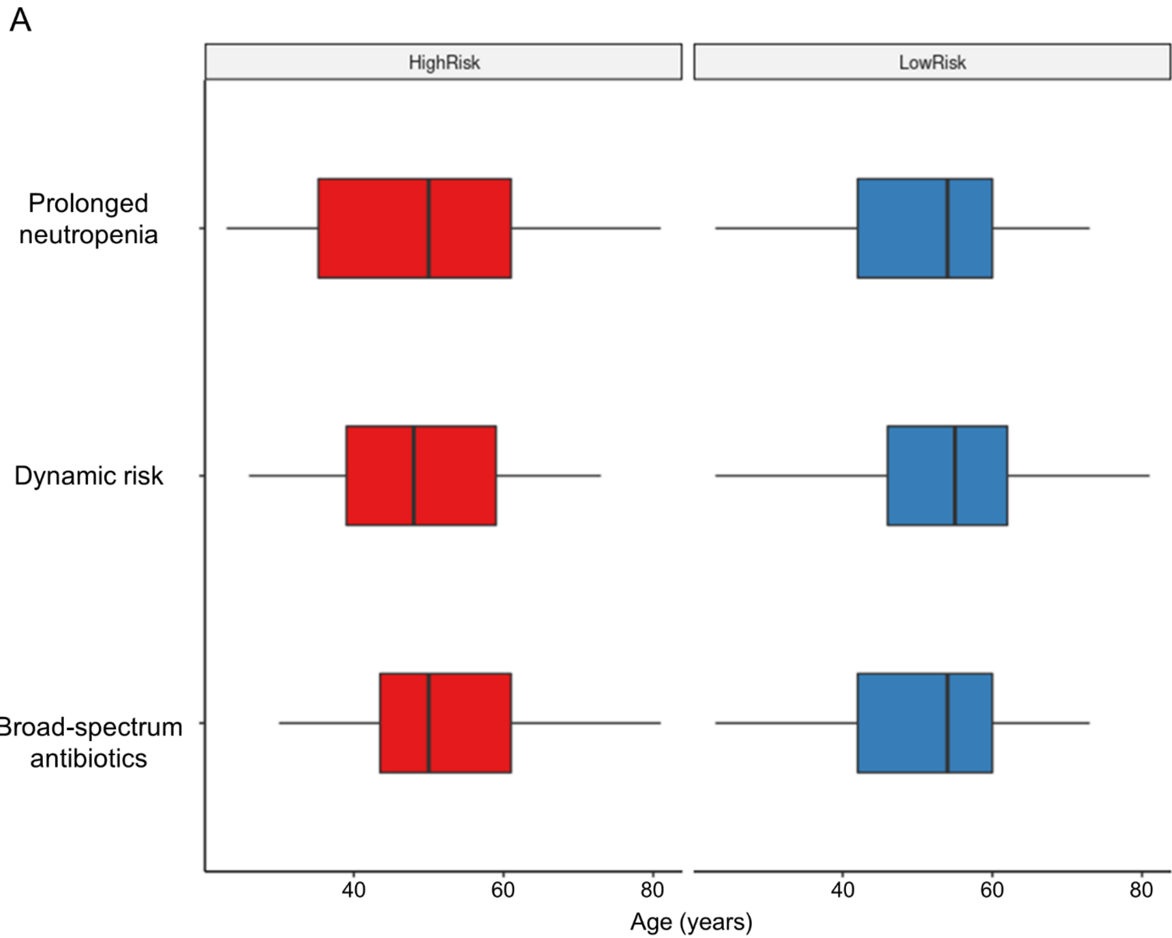
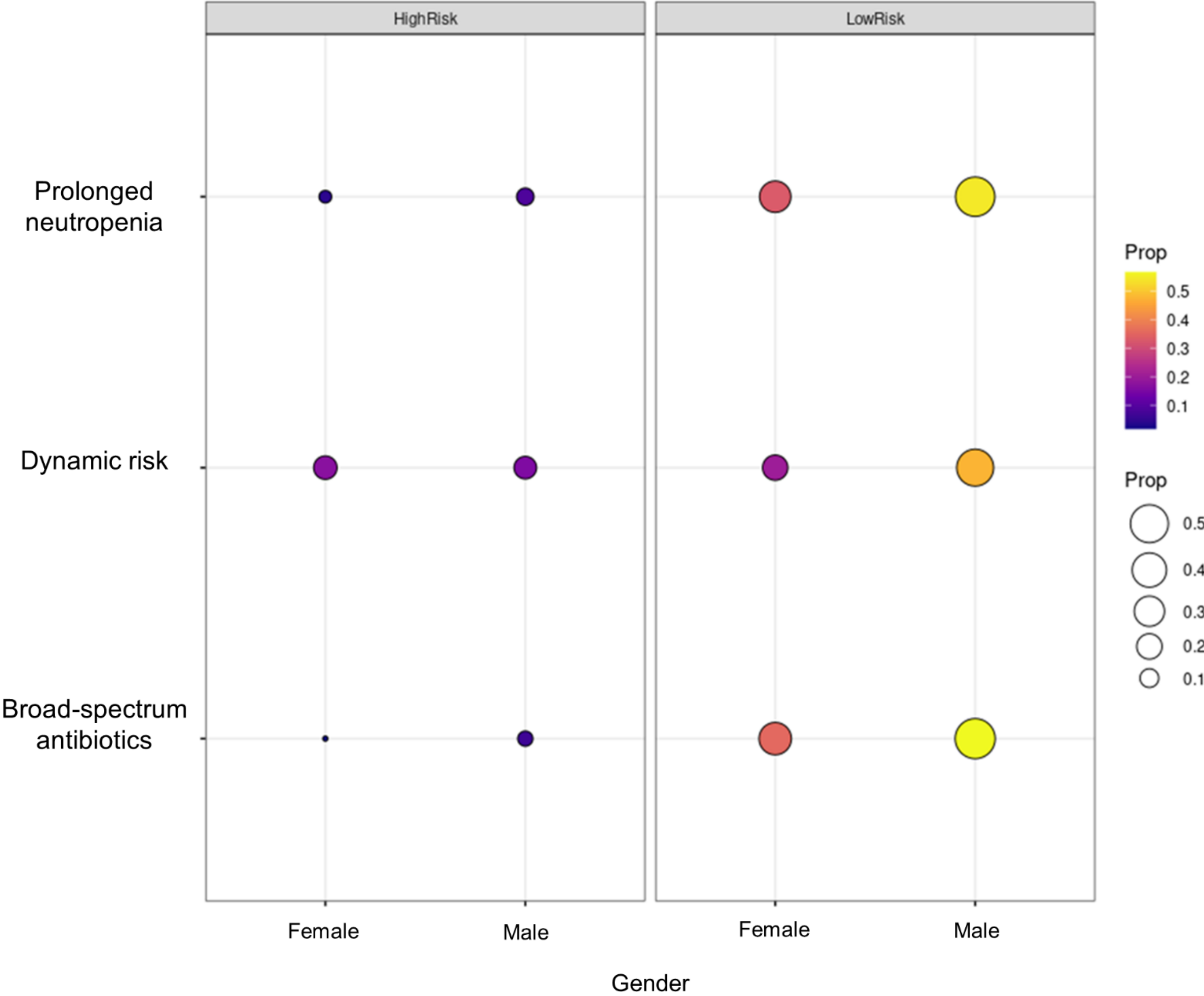


***Figure 2**. **The microbiome composition differs between LR and HR oropharyngeal samples**. **(A)** Boxplots of Observed OTUs, Chao1 and Shannon alpha diversity indexes grouped by risk of IFI according to three distinct criteria (presence or not of prolonged neutropenia, dynamic LR or HR according to SEIFEM algorithm, and use or not of broad-spectrum antibiotics). Significance was evaluated by applying a Kruskal-Wallis test (the p value is indicated). **(B)** Boxplots of Jaccard and Bray-Curtis beta diversity indexes evaluating distances within (LR, green; HR, red) or between (grey) LR and HR samples according to the three distinct criteria described in panel A. Significance was evaluated by applying a Kruskal-Wallis test (the p value is indicated).*

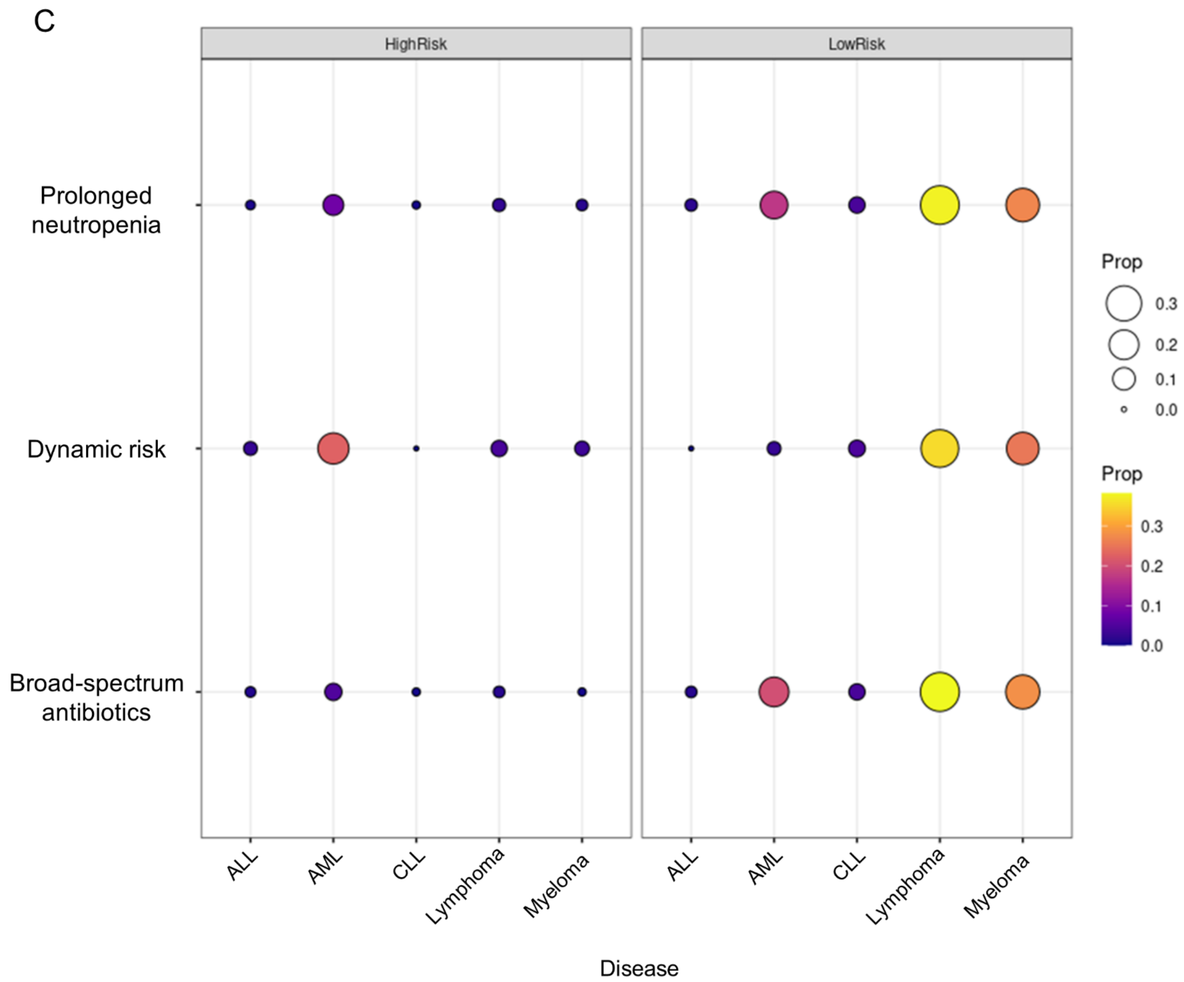
Supplementary Figure 1



Supplementary Figure 1

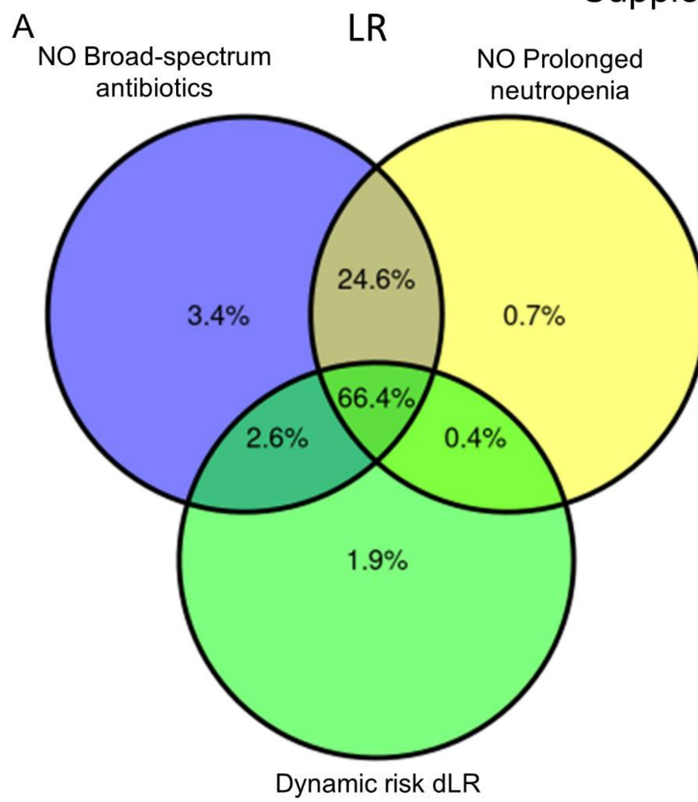


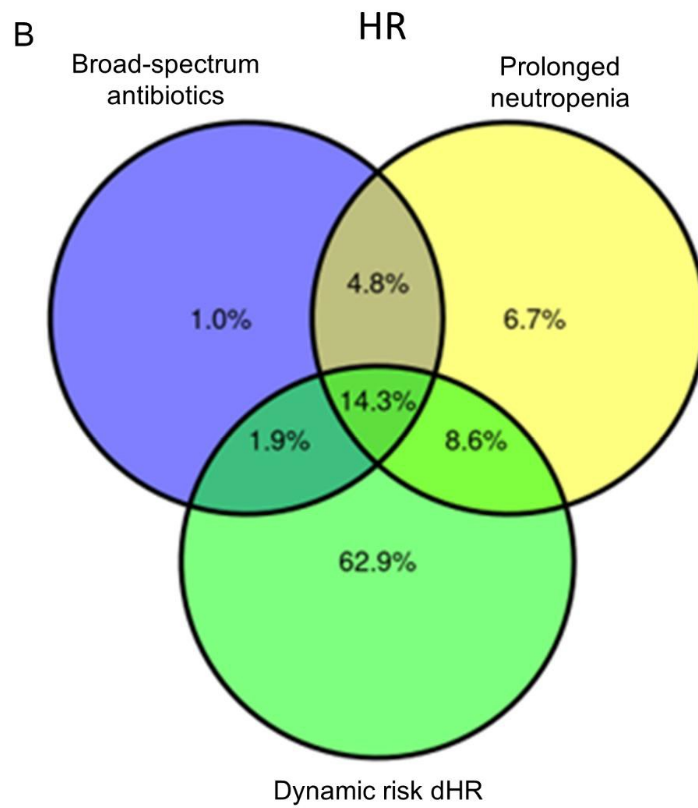
Supplementary Figure 1



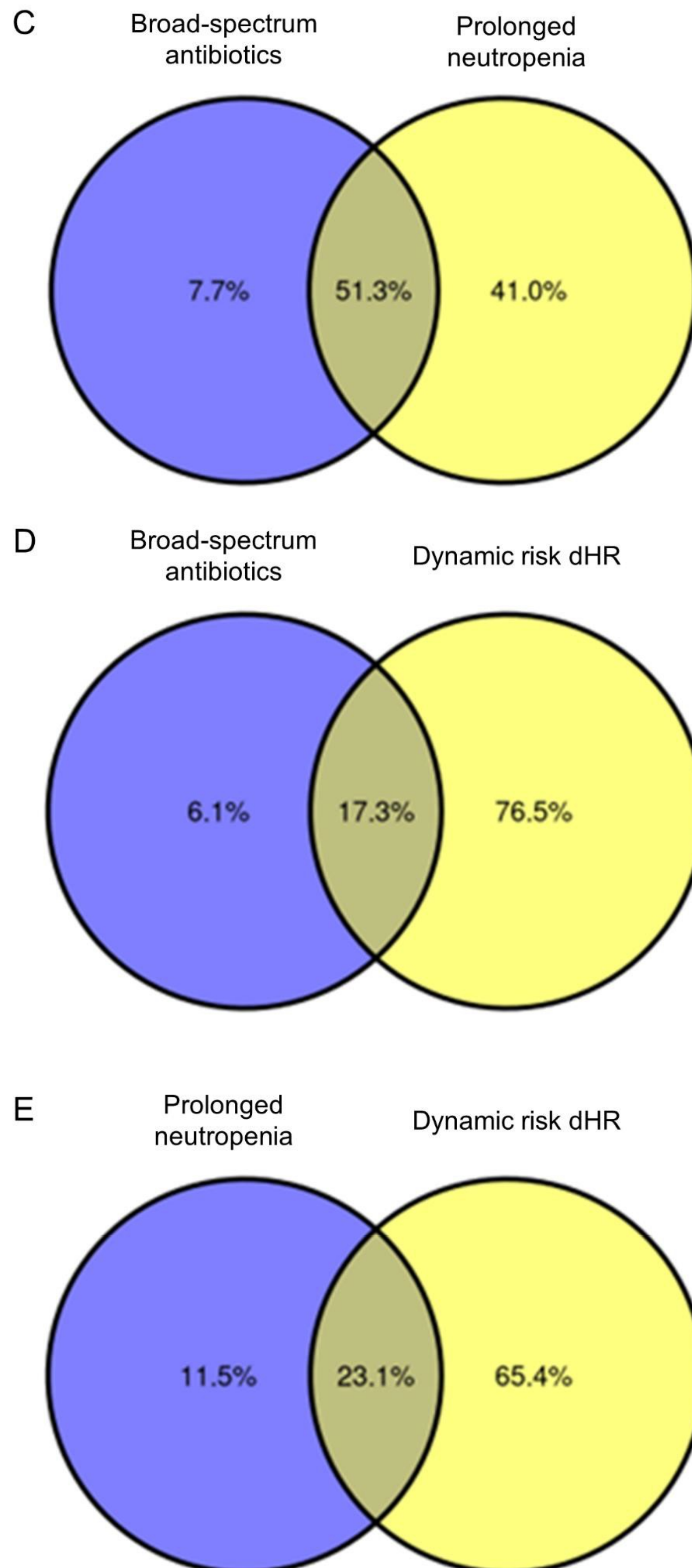
Supplementary Figure 1. HR and LR samples distribution obtained by applying each criterion for risk stratification separately. *In each plot, rows refer to the IFI risk criteria that define a High or Low Risk state for each sample. Within every criterion, boxplots of (**A**) show the distribution of patients age while each cell of the balloon plots in (**B**) and (**C**) contains a dot whose size reflects the relative magnitude of the corresponding proportion (sum of proportion of each row is equal to 1) according to gender (**B**) and hematological disease (**C**).*

Supplementary Figure 2



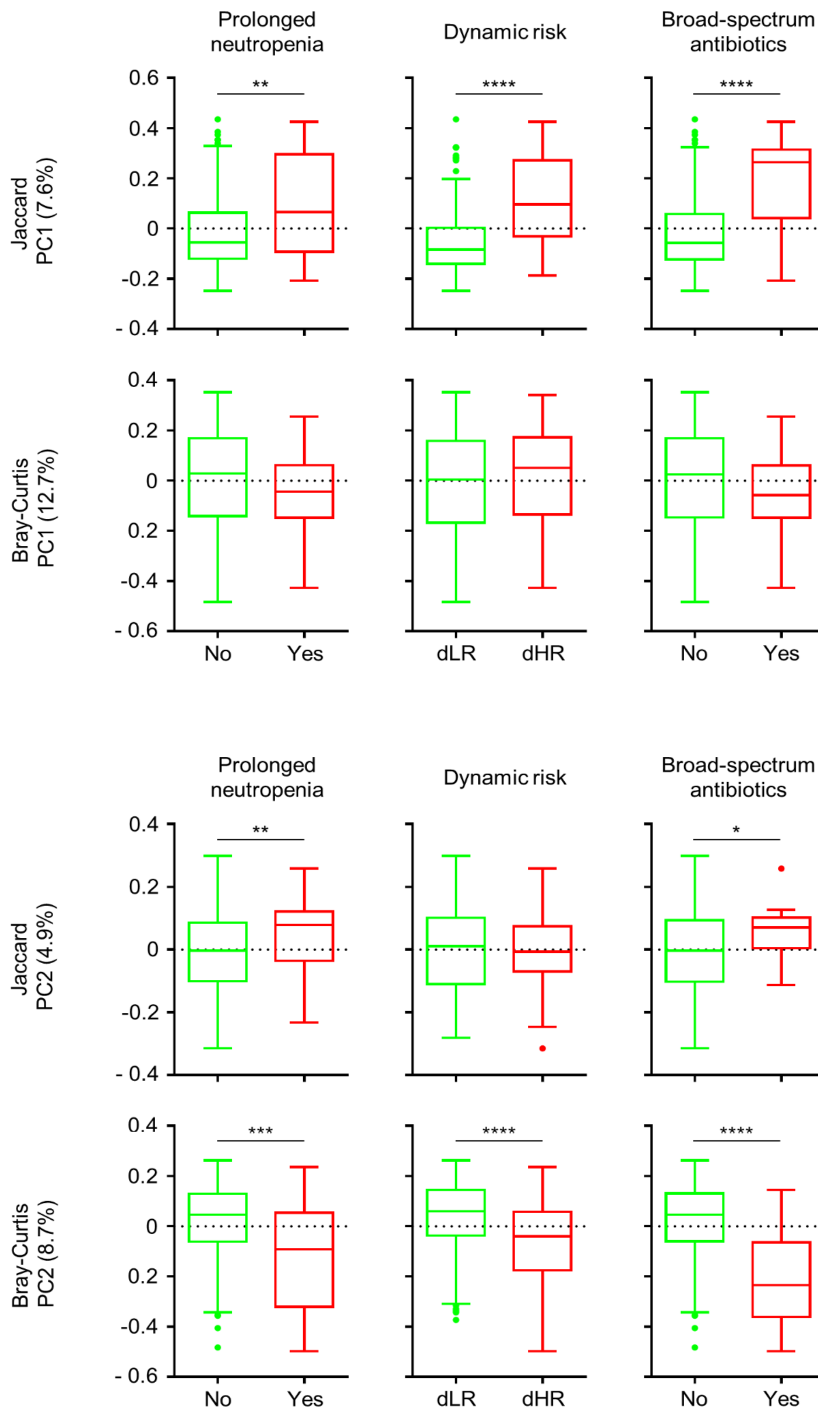


Supplementary Figure 2

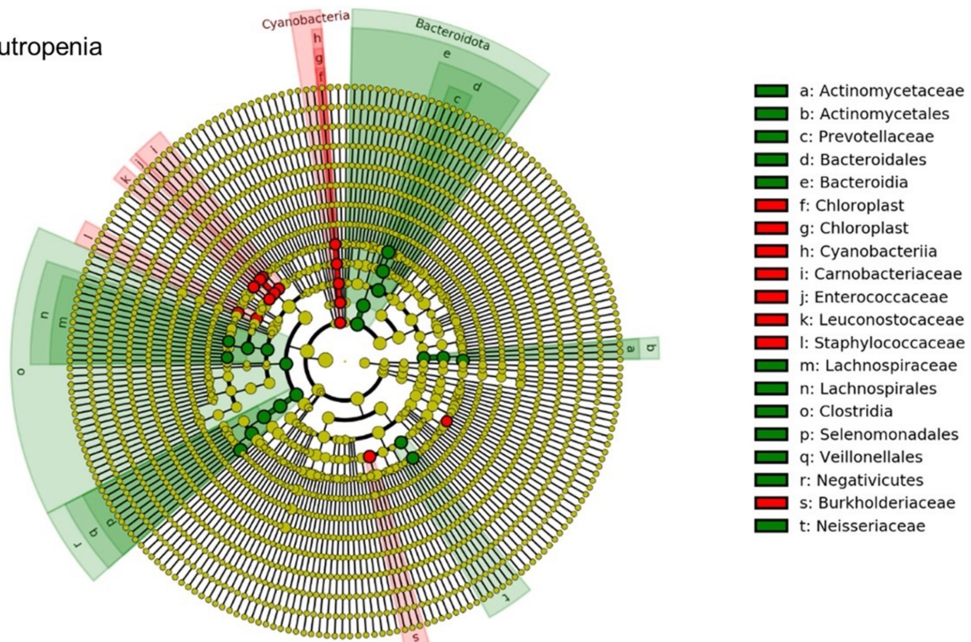


Supplementary Figure 2. Relationships between criteria for IFI risk. (A-B) *Venn diagrams show all possible logical relationships between low (**A**) and high (**B**) risk criteria, specifically, absence (**A**) or presence (**B**) of broad-spectrum antibiotics and prolonged neutropenia, and low (**A**) or high (**B**) dynamic risk. (**C-E**) Venn diagrams show all possible logical relationships between two high risk criteria in all possible combinations, i.e. administration of broad-spectrum antibiotics and presence of prolonged neutropenia (**C**), administration of broad-spectrum antibiotics and high dynamic risk (**D**), presence of prolonged neutropenia and high dynamic risk (**E**).*

Supplementary Figure 3



Supplementary Figure 3. The oropharyngeal microbiomes of LR and HR hematological patients display a different composition. *Boxplots of the first and second components of the Principal Coordinate Analysis (PCoA) of the Jaccard and Bray-Curtis indexes for the LR (green) and HR (red) samples according the three different criteria as described in Figure 2. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.*



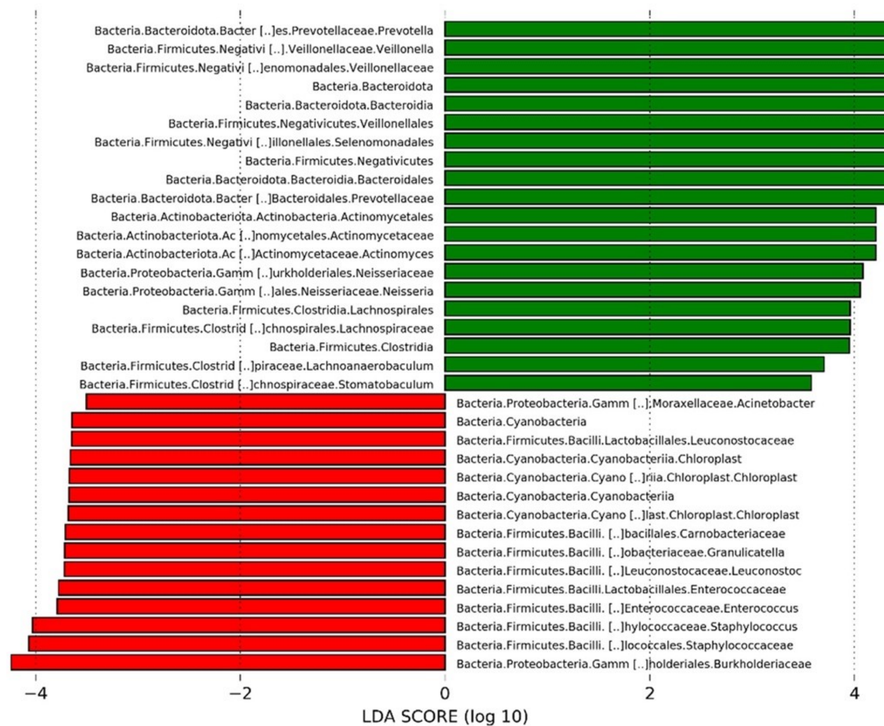


Figure 3. Oropharyngeal microbiomes have a different genus composition in function of prolonged neutropenia. Taxonomic visualization of statistically and biologically consistent differences between samples collected during the presence or absence of prolonged neutropenia. The cladogram simultaneously highlights high-level taxa and specific genera. Taxa (circles) are colored red when significantly (LEfSe, $P < 0.05$) associated with the presence of prolonged neutropenia, green when significantly associated with the absence of prolonged neutropenia, and yellow when not significantly associated with either group. The size of each circle is proportional to the abundance of the corresponding taxon in all samples. The histograms of the linear discriminant analysis (LDA) scores are computed for genera significantly associated with either the presence (red) or the absence (green) of prolonged neutropenia. LEfSe has been applied with default alpha values for the analysis of variance (ANOVA) and Wilcoxon tests (0.05), and the LDA effect size has been evaluated by setting the absolute value of the logarithmic LDA threshold equal to 3.5. Other LEfSe parameters have been set to the default.

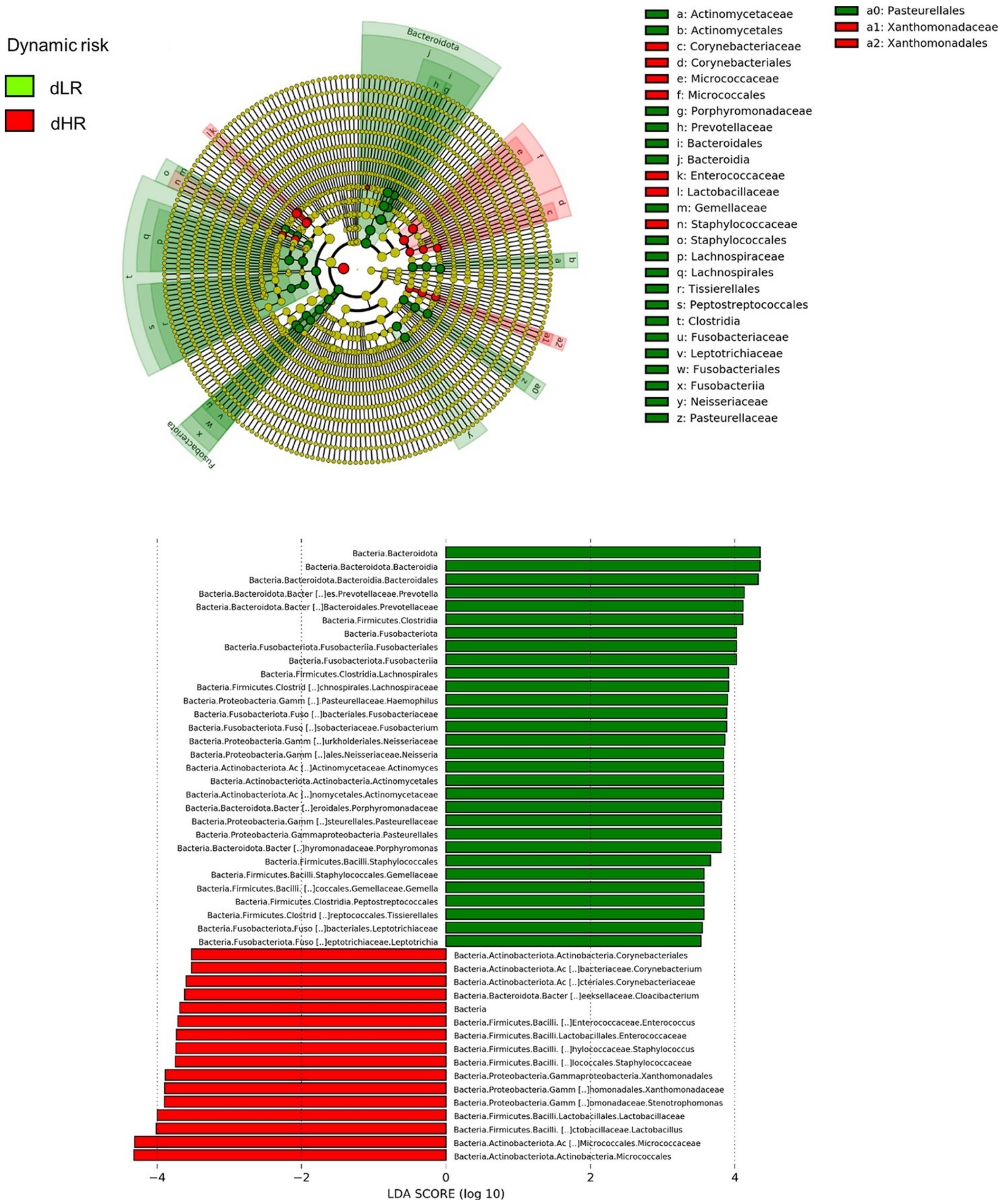
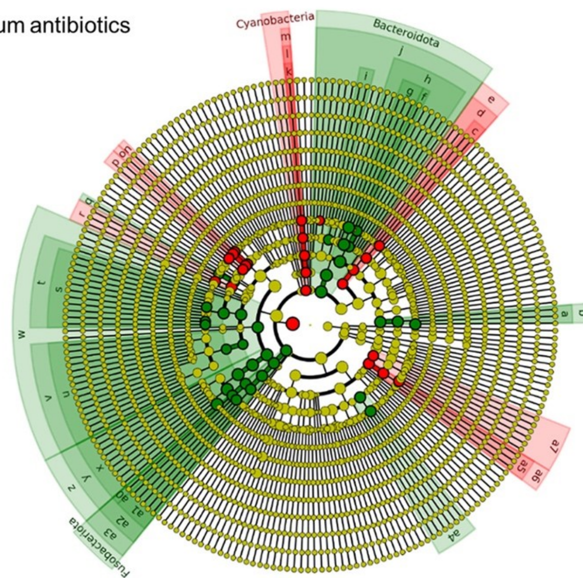


Figure 4. Oropharyngeal microbiomes have a different genera composition in function of dynamic risk stratification*. Taxonomic visualization of statistically and biologically consistent differences between samples allocated to low risk (dLR) or high risk (dHR) for IFI by dynamic risk stratification. The cladogram simultaneously highlights high-level taxa and specific genera. Taxa (circles) are colored red when significantly (LEfSe, P < 0.05) associated with dHR, green when significantly associated with dLR, and yellow when not significantly associated with either group. The size of each circle is proportional to the abundance of the corresponding taxon in all samples. The histograms of the LDA scores are computed for genera significantly associated with either dHR (red) or dLR (green) groups. LEfSe has been applied with default alpha values for the ANOVA and Wilcoxon test (0.05) and the LDA effect size has been evaluated by setting the absolute value of the logarithmic LDA threshold equal to 3.5. Other LEfSe parameters have been set to the default.*



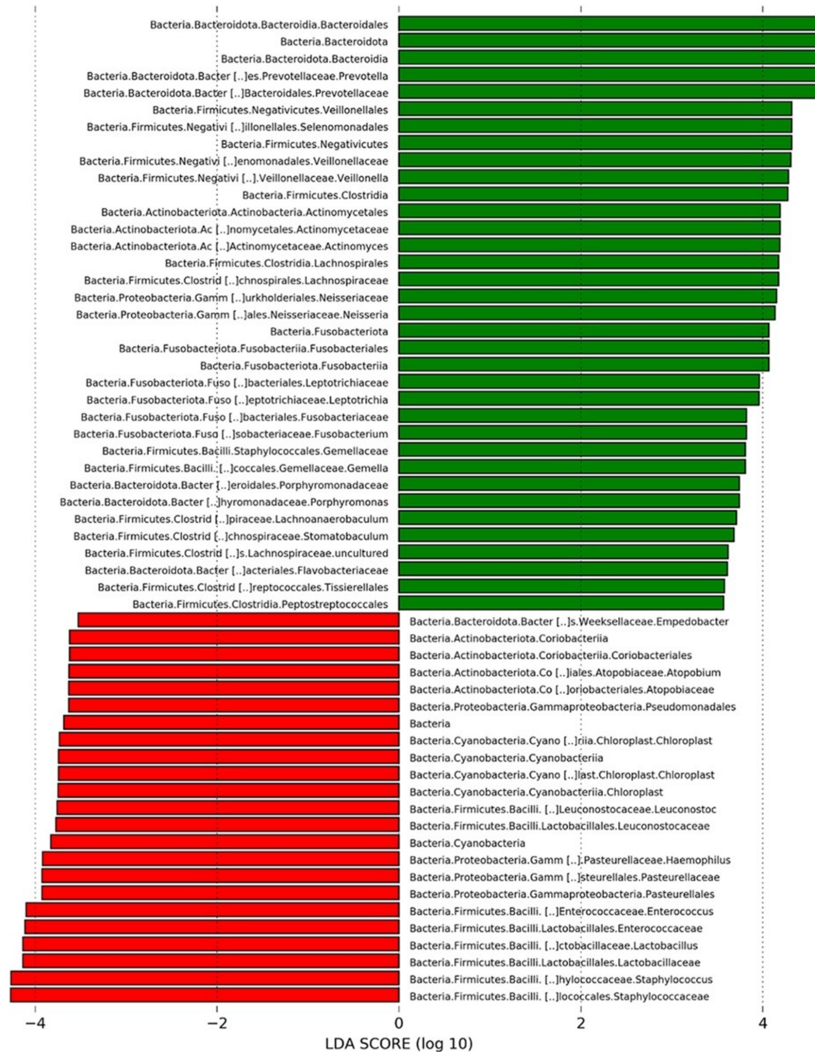


Figure 5 Oropharyngeal microbiomes have a different genera composition in function of broad-spectrum antibiotics use. Taxonomic visualization of statistically and biologically consistent differences between samples collected or not during treatments with broad-spectrum antibiotics. The cladogram simultaneously highlights high-level taxa and specific genera. Taxa (circles) are colored red when significantly (LEfSe, P < 0.05) associated with the use of broad-spectrum antibiotics, green when significantly associated with the absence of broad-spectrum antibiotics use, and yellow when not significantly associated with either group. The size of each circle is proportional to the abundance of the corresponding taxon in all samples. The histograms of the LDA scores are computed for genera significantly associated with either the use (red) or lack of use (green) of broad-spectrum antibiotics. LEfSe has been applied with default alpha values for the ANOVA and Wilcoxon test (0.05), and the LDA effect size has been evaluated by setting the absolute value of the logarithmic LDA threshold equal to 3.5. Other LEfSe parameters have been set to the default.

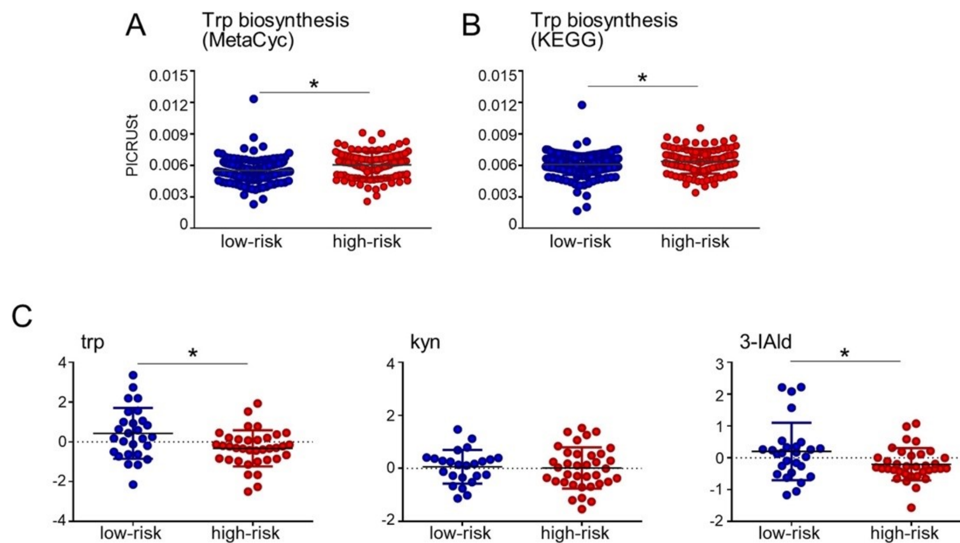
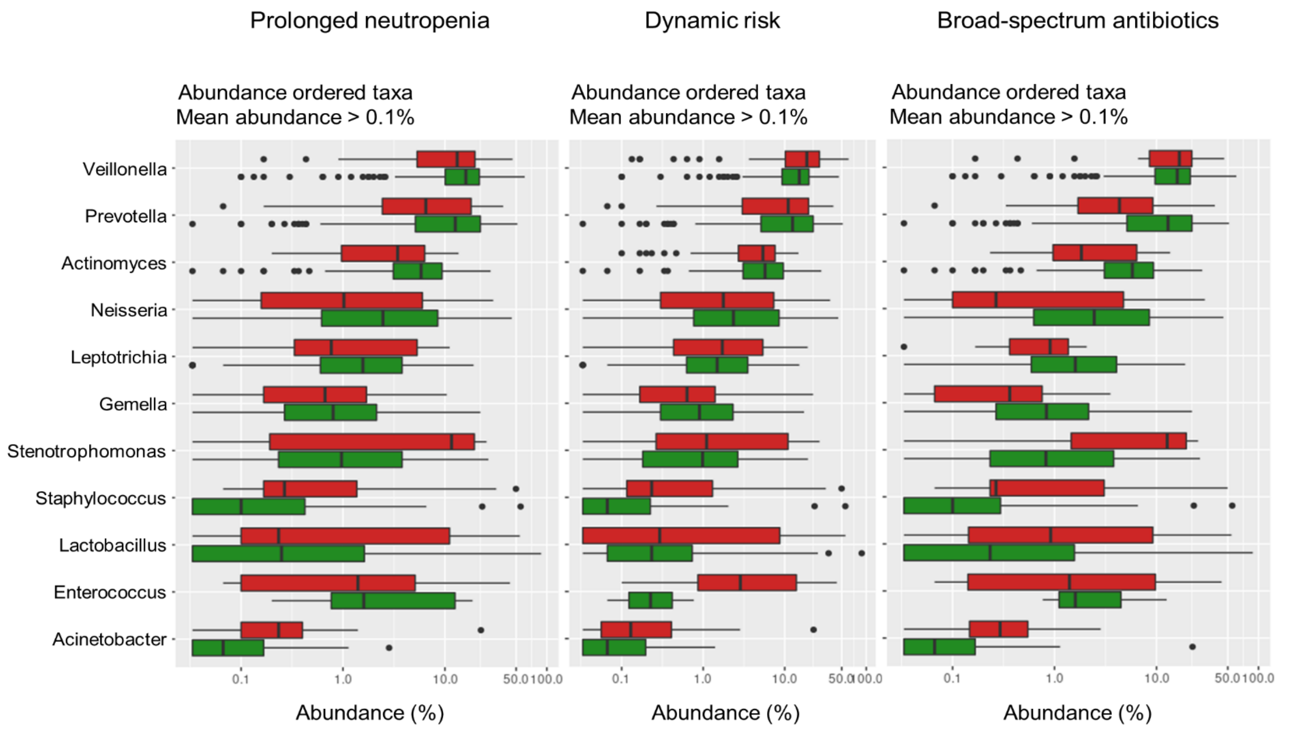


Figure 6. LR and HR oropharyngeal samples differ in tryptophan metabolism. (A and B) *Box plots of trp biosynthesis pathway (A) and module (B) inferred by PICRUSt2 analysis according to MetaCyc and KEGG databases, respectively. These predicted metagenome functions were indicated by LEfSe as significantly differentially represented in the high- and low-risk groups*. ***(C)*** *Tryptophan (trp), kynurenines (kyn), and indole-3-aldehyde (3-IAld) levels (nmol/liter) were measured in oropharyngeal samples (n = 63; LR, 27; HR, 36) and expressed as means ± standard deviations (SD). *, P < 0.05 LR versus HR, unpaired t test*

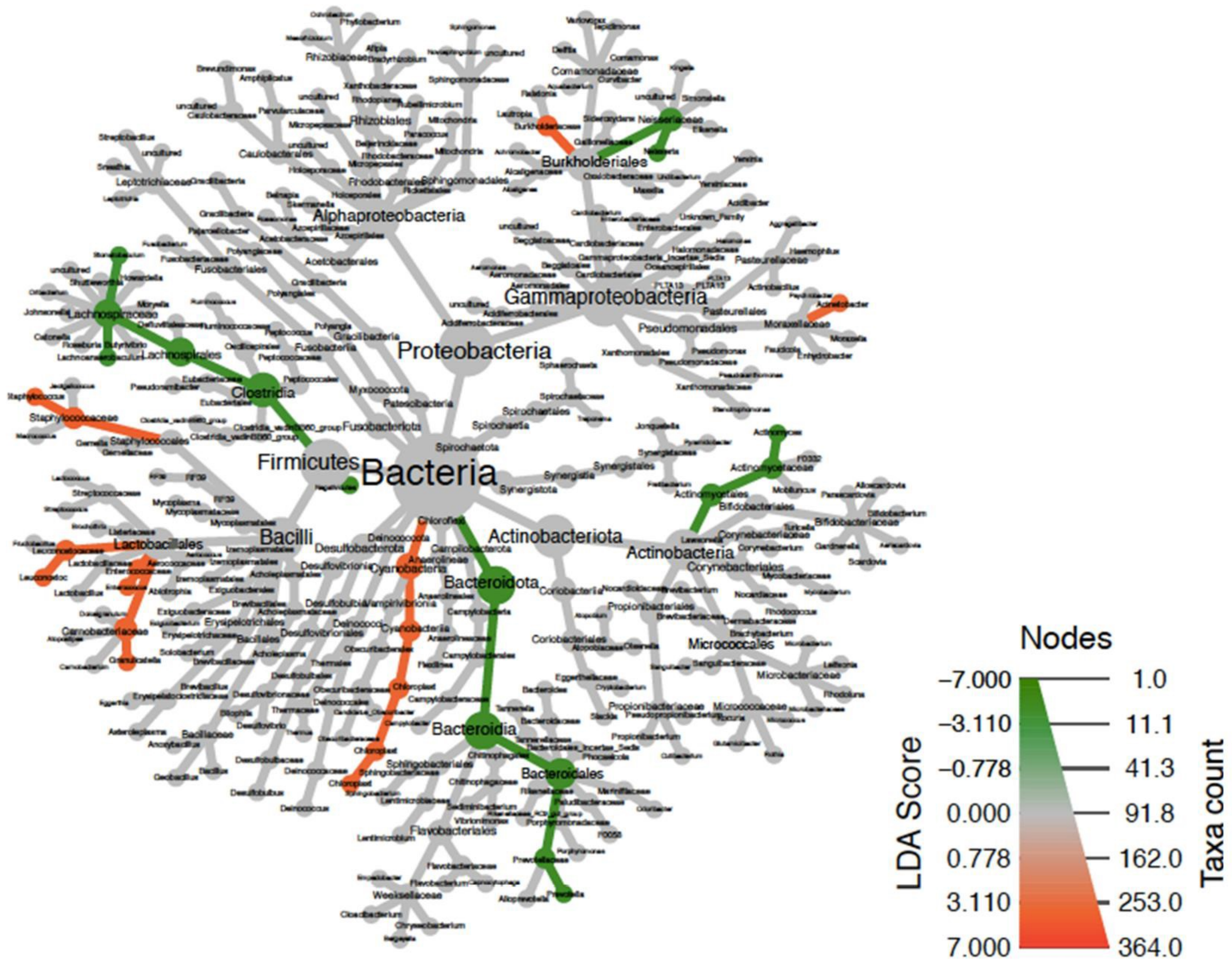
Supplementary Figure 4



Supplementary Figure 4. The oropharyngeal microbiome of the LR and HR samples are enriched with different genera. *Boxplots of the genera that LEfSE significantly associates to either the LR (green) or the HR (red) groups according to the three different criteria as described in Figure 2.*

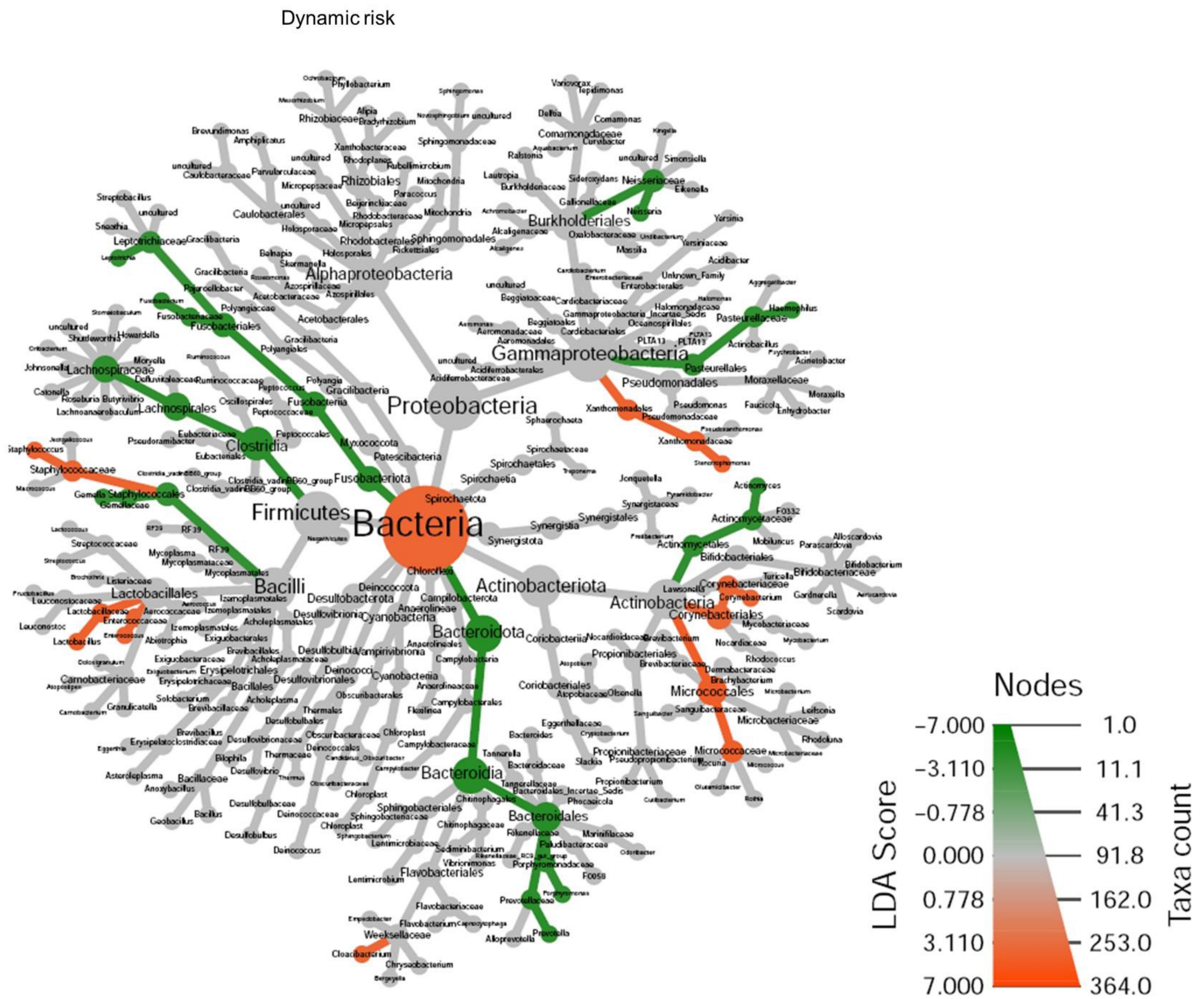
Supplementary Figure 5A

Prolonged neutropenia



Supplementary Figure 5. The oropharyngeal microbiomes of the LR and HR samples have a different composition at the genus level. *LEfSe results reported on a taxonomic heat tree up to genus level. Green-colored and red-colored nodes indicate bacteria significantly associated with LR (green) and HR (red) samples, respectively, according to the three criteria as described in Figure 2.*

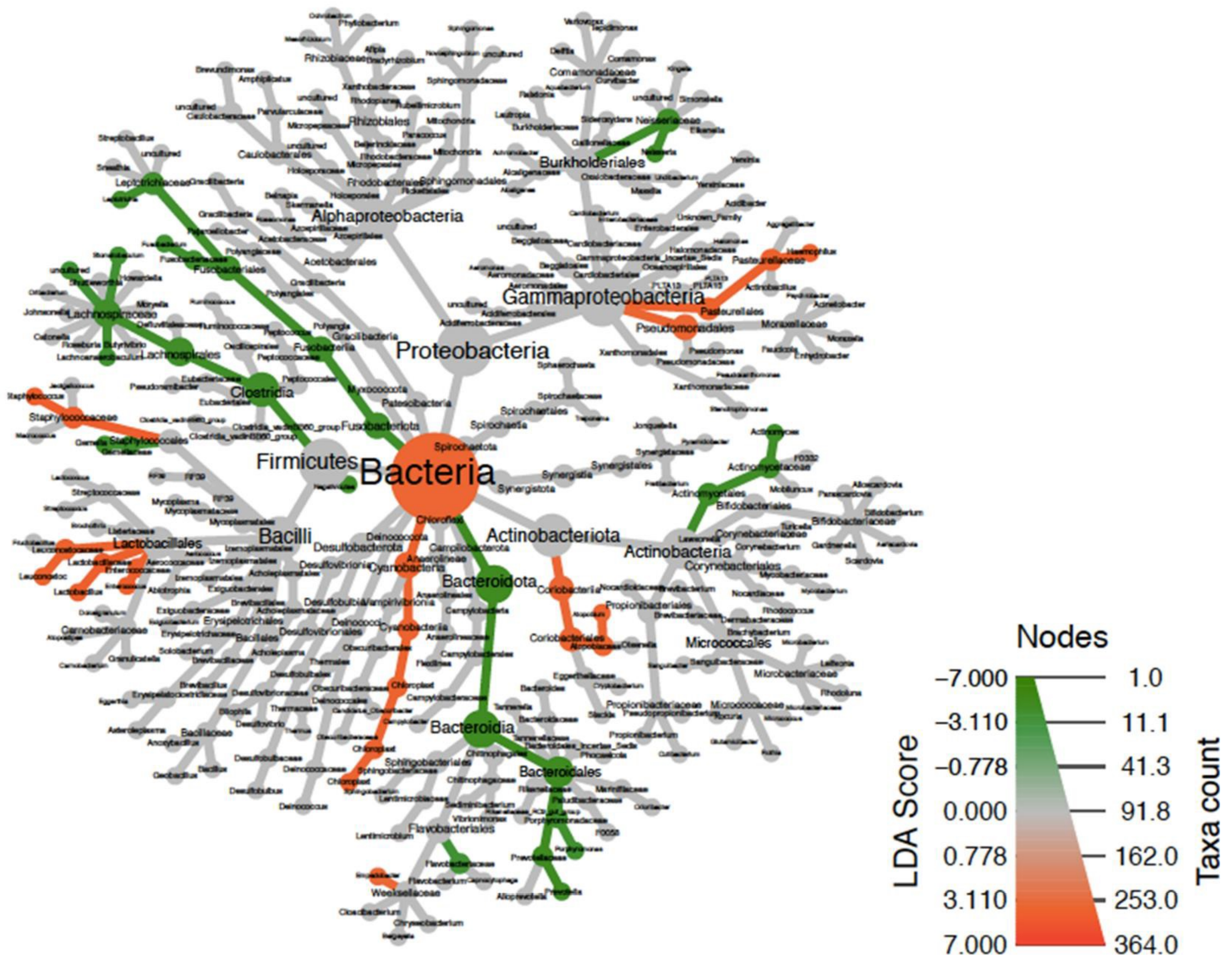
Supplementary Figure 5B



Supplementary Figure 5. The oropharyngeal microbiomes of the LR and HR samples have a different composition at the genus level. *LEfSe results reported on a taxonomic heat tree up to genus level. Green-colored and red-colored nodes indicate bacteria significantly associated with LR (green) and HR (red) samples, respectively, according to the three criteria as described in Figure 2*

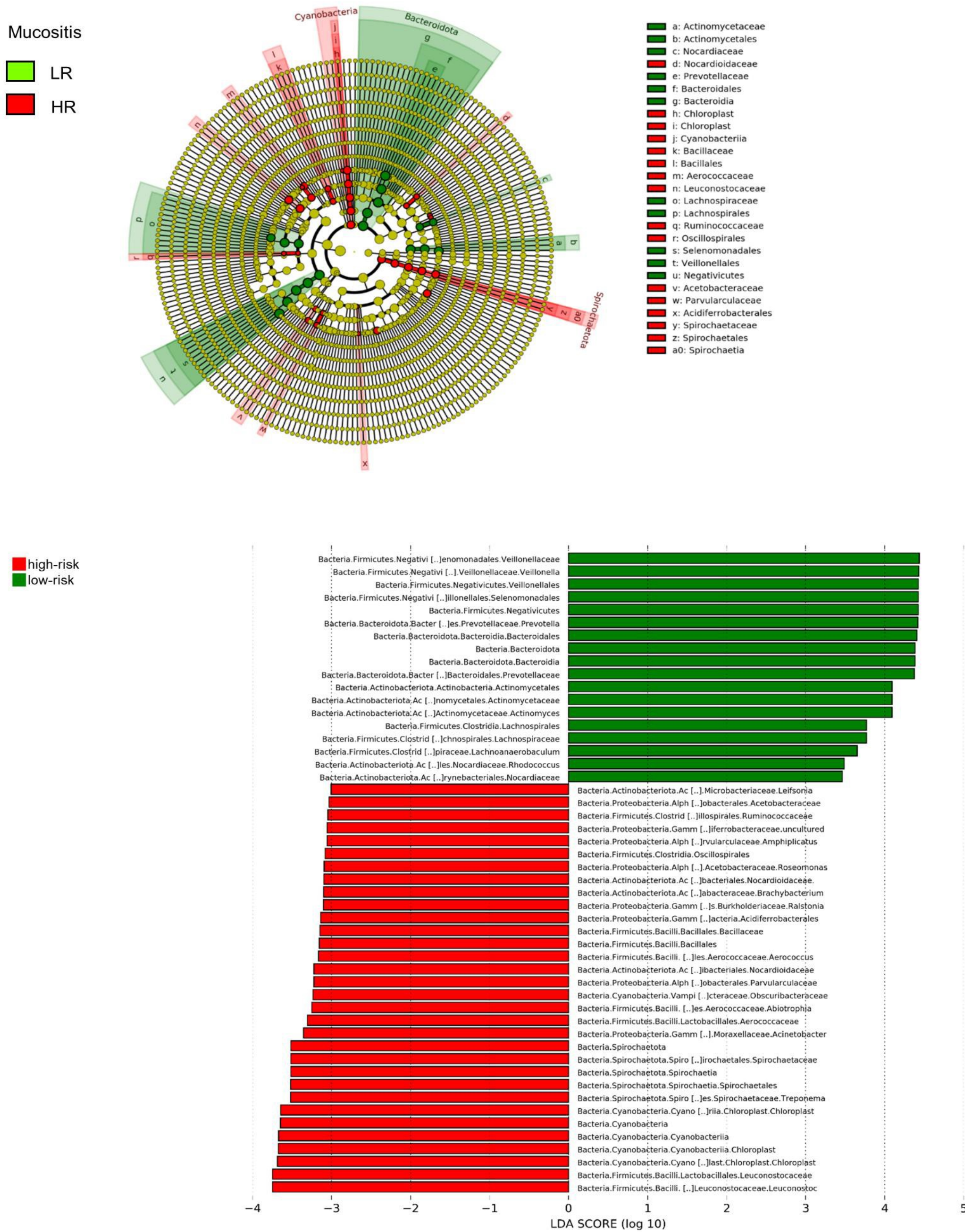
Supplementary Figure 5C

Broad-spectrum antibiotics



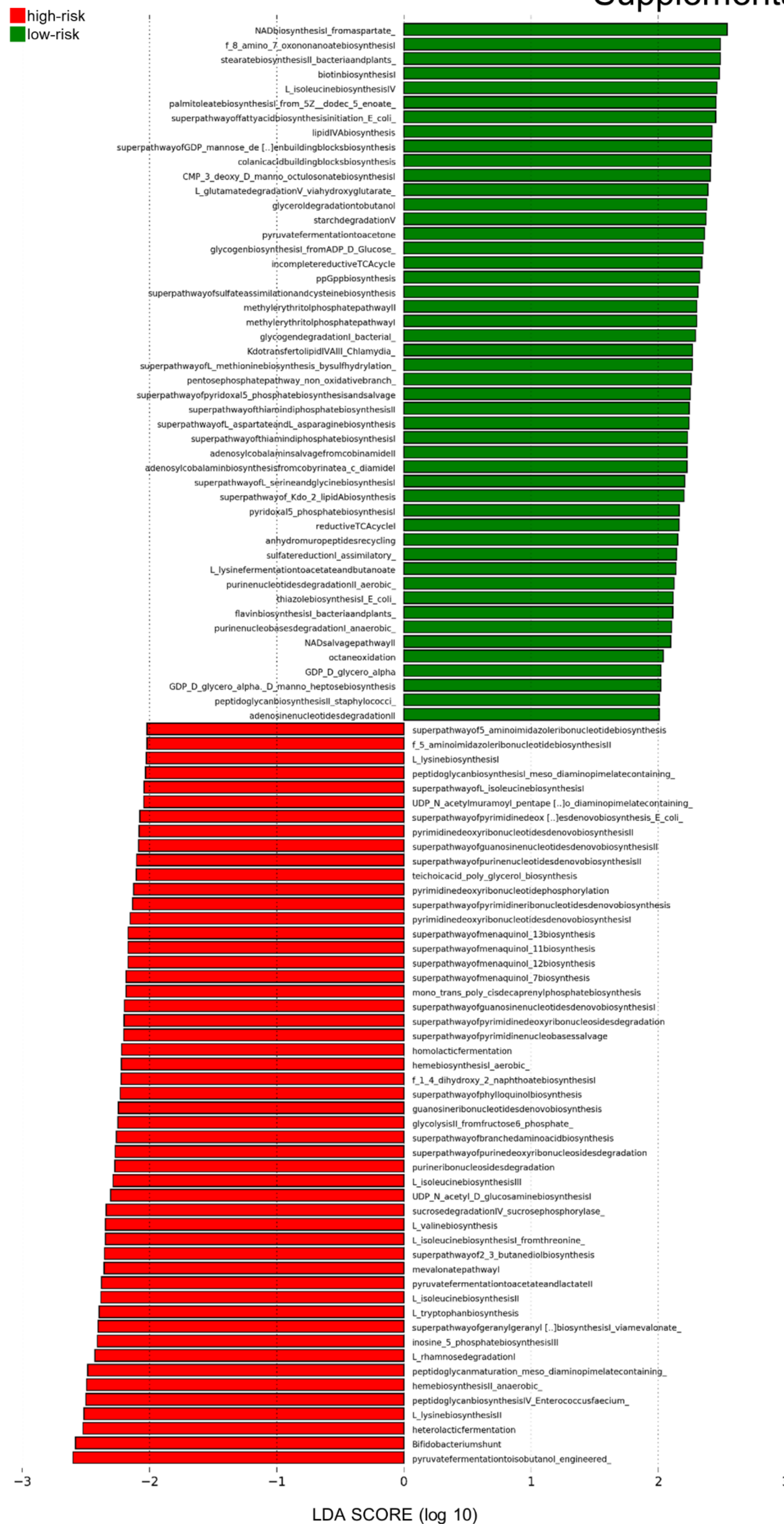
Supplementary Figure 5. The oropharyngeal microbiomes of the LR and HR samples have a different composition at the genus level. *LEfSe results reported on a taxonomic heat tree up to genus level. Green-colored and red-colored nodes indicate bacteria significantly associated with LR (green) and HR (red) samples, respectively, according to the three criteria as described in Figure 2.*

Supplementary Figure 6



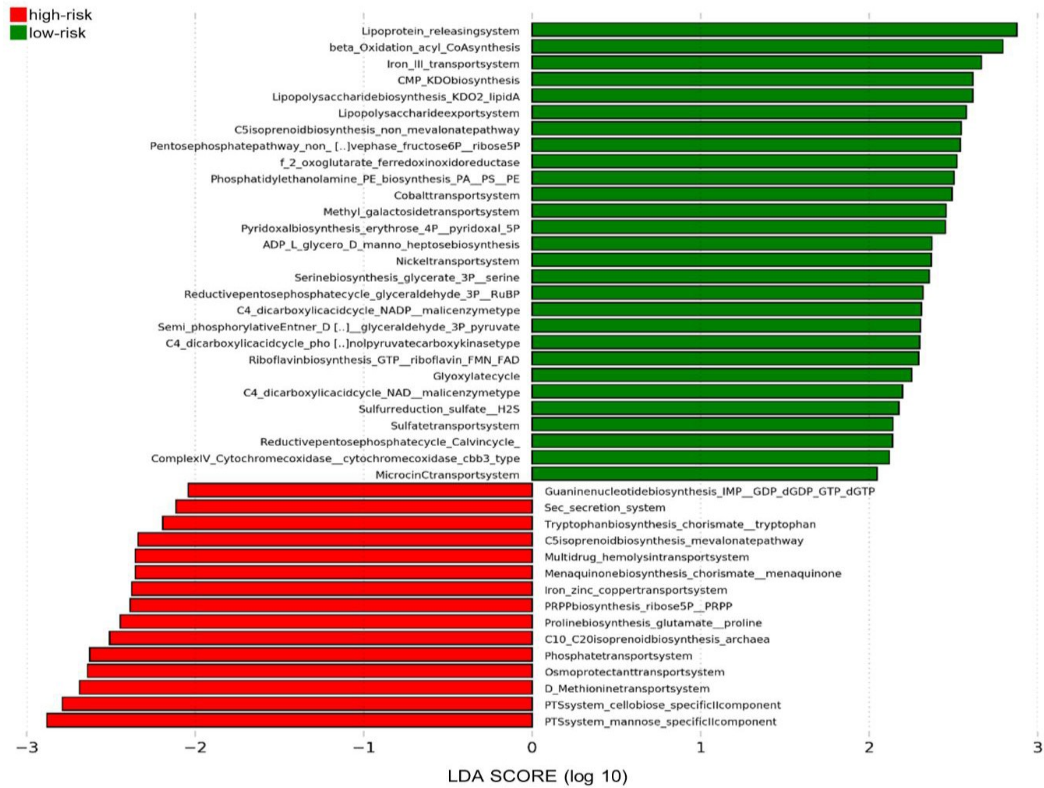
Supplementary Figure 6. The oropharyngeal microbiomes have a different genera composition in function of mucositis. *Taxonomic visualization of statistically and biologically consistent differences between samples collected during the presence or absence of mucositis. The cladogram simultaneously highlights high-level taxa and specific genera. Taxa (circles) are colored red when significantly (LEfSe , P< 0.05) associated to the presence of mucositis, green when significantly associated to the absence of mucositis, and yellow when not significantly associated to either group. The size of each circle is proportional to the abundance of the corresponding taxon in all samples. The histograms of the LDA scores are computed for genera significantly associated to either the presence (red) or the absence (green) of mucositis. LEfSe has been applied with default alpha values for the Anova and Wilcoxon test (0.05) and the LDA effect size has been evaluated by setting the absolute value of the logarithmic LDA threshold equal to 3.5. Other LEfSe parameters have been set to the default.*

Supplementary Figure 7



Supplementary Figure 7. The oropharyngeal microbiomes of the LR and HR samples have a different metabolic profile according to MetaCyc database. *Histogram of the LDA scores computed on metabolic functions inferred by PICRUSt2 analysis significantly associated to either LR or HR groups. The threshold value of the logarithmic LDA score was set to 2.0*

Supplementary Figure 8



Supplementary Figure 8. The oropharyngeal microbiomes of the LR and HR samples are enriched with different metabolic pathways according to KEGG modules. *Histogram of the LDA scores computed on metabolic functions inferred by PICRUSt2 analysis significantly associated to either LR or HR groups. The threshold value of the logarithmic LDA score was set to 2.0.*

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