COVID-19 severity and complications associated with low diversity, dysbiosis and predictive metagenome features of the oropharyngeal microbiome.

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Letter

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Abstract

Coronavirus 2 (SARS-CoV-2) infection and the resulting COVID-19 illness vary from asymptomatic disease, mild upper respiratory tract infection, pneumonia¹, to a life-threatening multi-organ failure with case fatality rates ranging from 0.27–13.4%^{2,3}. Despite increasing knowledge of the clinical and immunological features underlying COVID-19^{1,4–6}, biological variables explaining the course of infection and its severity remain elusive. At the entry site of SARS-CoV2, the oropharyngeal microbiome represents a hub integrating viral and immune signals at the start of the infection^{7–10}. To evaluate the role of the oropharyngeal microbiome in COVID-19, we performed a multi-center, cross-sectional clinical study analyzing the oropharyngeal microbial metagenomes in healthy adults, patients with non-SARS-CoV-2 infections, or with mild, moderate and severe COVID-19 encompassing a total of 345 participants. Significantly reduced microbiome diversity and high dysbiosis were observed in hospitalized patients with severe COVID-19, which was further associated with a loss of microbial genes and metabolic pathways. In this cohort, diversity measures were also associated with need for intensive care treatments as major clinical parameters in COVID-19. We further applied random forest machine learning to unravel microbial features for segregating clinical outcomes in hospitalized cases, and observed oropharyngeal microbiome abundances of *Haemophilus* or *Streptococcus* species as most important features. These findings provide insights into the role of the oropharyngeal microbiome in SARS-CoV-2 infection, and may suggest new biomarkers for COVID-19 severity.

Main Text

The microbiome constitutes a signaling hub regulating host immunity, mucosal homeostasis and defense against pathogens⁷. The oropharyngeal and lung microbiome has been previously studied during respiratory syncytial virus (RSV) and influenza respiratory tract infection^{8–10}. At these body sites, the microbiome was found to modulate viral infections through adaptations of mucosal immunity or inhibition of mucosal virus adherence¹¹, which eventually affect the clinical course of these infections¹². Viruses can also facilitate pathogen colonization, eventually leading to bacterial superinfections and acute respiratory distress syndrome^{13,14}. Therefore, we hypothesized that the presence of a specific microbial ecology may restrict or facilitate SARS-CoV-2 infection and determine the severity and clinical course of COVID-19.

To study the role of the human oropharyngeal microbiome in SARS-CoV-2 infection and illness, we collected oropharyngeal biospecimens from a multi-center patient and healthy volunteer cohort in Germany (n=345). Between 30 March and 20 May 2020, we recruited healthy adult individuals, patients with mild, moderate or severe COVID-19, and SARS-CoV-2 negative patients with mild upper respiratory infections or with critical pneumonia. Demographic and clinical characteristics are depicted in Table 1. Mild COVID-19 was assigned to patients presenting with symptoms of upper respiratory tract infections that were not hospitalized (Extended Data Fig. 1). Patients with moderate or severe disease were hospitalized with lower respiratory tract symptoms. In our cohort, 66 cases with severe COVID-19 required

intensive care unit (ICU) therapy, and 41 out of them deteriorated over the course of hospitalization and required invasive mechanical ventilation or extracorporeal membrane oxygenation (ECMO). Fifteen hospitalized patients (17.2%) died by the conclusion of our study. Extended Data Fig. 2 presents medical treatments and pre-existing conditions of these patients.

Table 1. Baseline demographic characteristics of healthy subjects, individuals with an upper respiratory tract (URT) infection, or with COVID-19. Significant effect of age across all group (Kruskal-Wallis test: $p < 0.0001$); IQR, inter-quartile range.

	Healthy individuals	URT infection	Mild COVID-19	Moderate COVID-19	Severe COVID-19
Subjects	n = 74	n = 112	n = 36	n = 27	n = 66
SARS-CoV-2 status	negative	negative	positive	positive	positive
Age					
Median (IQR) – yr	36.0 (29.75 - 53.25)	46.0 (31.0 - 57.0)	50.0 (36.75 - 55.5)	57.0 (46.25 - 72.75)	64.0 (53.25 - 72.75)
Distribution – no.(%)					
18 – 49 yr	54 (73.0)	64 (57.1)	17 (47.2)	7 (25.9)	9 (13.6)
50 – 64 yr	19 (25.7)	36 (32.1)	17 (47.2)	12 (44.4)	23 (34.8)
≥ 65 yr	1 (1.4)	12 (10.7)	2 (5.6)	8 (29.6)	28 (42.4)
Gender – no.(%)					
Female	48 (64.9)	75 (67.0)	24 (66.7)	10 (27.0)	14 (21.2)
Male	26 (35.1)	37 (33.0)	12 (33.3)	17 (62.9)	46 (69.7)

To characterize the oropharyngeal microbiome profiles in our cohort, we used shotgun metagenome sequencing on oropharyngeal samples from all 345 study subjects that were collected either during SARS-CoV-2 screening of suspected cases or during/after admission of patients with confirmed or suspected COVID-19 to one of the seven medical centers participating in this study. Moderate and severe COVID-19 was associated with a significant decrease or loss of the oropharyngeal bacterial diversity (Fig. 1a). Differences in microbiome compositions between each group were most apparent in a principle-coordinate analysis (PCoA) with significant separations according to COVID-19 severity (PERMANOVA: $F=23.15$, $p=0.002$; Fig.1b, Extended Data Fig. 3a for t-SNE). Color-coding the PCoA projection according to diversity separated low vs. high diversity states (Extended Data Fig. 3b). We further classified samples with taxonomic compositions highly unlike those of healthy control samples as “dysbiotic”¹⁵ as measured by Bray-Curtis beta-diversity distances¹⁶ (Fig. 1c) and observed that dysbiosis contributed significantly to the variation of the oropharyngeal microbiome (PERMANOVA $F=42.8$; $p=0.002$). Patients with moderate and severe COVID-19 showed a significantly higher dysbiosis index of the oropharyngeal microbiome compared to healthy controls, URT patients and mild COVID-19 patients, respectively (Fig. 1d). Antibiotic exposure and age had moderate effects on compositional diversity in the PCoA (Extended Data Fig. 3c, d). Patients admitted to the ICU because of severe respiratory distress other than COVID-19 also showed significantly lower diversity and higher dysbiosis indices of their oropharyngeal microbiome (Extended Data Fig. 4, 5 including clinical characteristics).

A highly diverse ecology of metagenomic species was observed in healthy controls, patients with non-SARS-CoV-2 URT or mild COVID-19 (Fig. 2a). To better understand the microbiome patterns across the different infection and disease states, we quantified the taxonomic groups that contributed to mono-

domination events, which are defined by a relative-abundance threshold of any single species of at least 30%¹⁶. As presented in Extended Data Fig. 6, *Staphylococcus aureus*, *Rothia mucilaginosa* and *Enterococcus faecium* were highly enriched species in patients with moderate and severe COVID-19, whereas *Prevotella spp.* were found to mono-dominate the oropharyngeal microbiome of healthy subjects and patients with mild infections. Hypothesizing the oropharyngeal microbiome is associated with disease severity, we investigated the taxonomic profiles of patients with mild vs. severe or moderate vs. severe COVID-19 and found *Prevotella*, *Streptococcus* and *Haemophilus spp.* to significantly discriminate these groups (Figure 2b; Supplemental Table with a complete list of taxa). Variance in the species profiles of hospitalized patients was only partly explained by antibiotic administration, but not by age and gender (Extended Data Fig. 7). Next, we analyzed genes and pathways of the oropharyngeal microbiome in our cohort, and found several microbial genes and pathways to be significantly enriched in moderate vs. severe disease, high vs. low alpha diversity, or low vs. high dysbiosis (Fig. 2c). Similar to the differences in taxonomy, a loss of genes and pathways, notably transposases, amino acid and DNA biosynthesis or sugar degradation pathways, characterized severe COVID-19, low diversity and high dysbiosis, as depicted in Fig. 2d.

In addition to characterizing the oropharyngeal microbiome across different COVID-19 severity states, we also aimed to study associations between individual microbiome features of the oropharyngeal microbiome and selected clinical parameters or outcomes. As diversity measure are increasingly used as microbial biomarkers for disease outcomes¹⁶, we assessed the associations of Shannon diversity and dysbiosis scores with treatment categories and outcomes in hospitalized patients using logistic regression analyses. ICU admission and need for mechanical ventilation or for ECMO therapy were significantly associated with microbial diversity or dysbiosis (Table 2). The association of dysbiosis and mechanical ventilation or ECMO therapy remained significant after multivariable adjustment for age, gender, center, antibiotic and steroid treatment (Table 2).

Table 2. Associations of microbiome diversity and dysbiosis with clinical outcomes. *Top*, univariate analyses applying a logistic regression analysis revealed significant associations of alpha diversity (Shannon index) and dysbiosis with ICU admission, mechanical ventilation and ECMO treatment in the group of all hospitalized, moderate and severe COVID-19 patients. *Bottom*, multivariate logistic regression testing adding age, gender, center, antibiotic and steroid treatment as covariates; dysbiosis index remained a significant predictor of mechanical ventilation and ECMO after this adjustment. OR, odds ratio; CI, confidence interval.

Univariate analyses																				
Predictors	Outcomes	OR (95% CI)		p-values																
Shannon index	ICU admission	0.48 (-1.25,-0.25)		0.005																
	Mechanical ventilation	0.48 (-1.19,-0.27)		0.002																
	ECMO	0.52 (-1.21,-0.10)		0.021																
	Mortality	1.11 (-0.43,0.64)		0.706																
Dysbiosis index	ICU admission	969.4 (2.8, 10.9)		< 0.001																
	Mechanical ventilation	7.7e5 (4.1, 5.13, 75)		< 0.001																
	ECMO	3.6e4 (2.3, 2.18, 68)		0.012																
	Mortality	12.32 (-2.53, 7.55)		0.329																
Multivariate analyses																				
Outcomes	ICU admission	OR (95% CI)		p-values		Mechanical ventilation	OR (95% CI)		p-values		ECMO	OR (95% CI)		p-values		Mortality	OR (95% CI)		p-values	
Covariates	Shannon index	0.64 (-1.07,0.18)		0.165		Shannon index	0.68 (-0.9,0.15)		0.145		Shannon index	0.49 (-1.5,0.05)		0.68		Shannon index	1.44 (-0.240, 97)		0.237	
	Age	1.01 (-0.03,0.046)		0.821		Age	1.02 (-0.01,0.06)		0.227		Age	0.97 (-0.08,0.02)		0.25		Age	1.07 (0.012,0.12)		0.018	
	Gender	1.99 (-0.51,1.88)		0.252		Gender	1.49 (-0.79,1.59)		0.515		Gender	3.5 (-0.44,2.94)		0.147		Gender	7.01 (-0.224, 12)		0.079	
	Study center	0.50 (-1.91,0.52)		0.276		Study center	2.05 (-0.4,1.36)		0.221		Study center	2.29 (1.1,5.2)		0.003		Study center	1.53 (-0.91, 75)		0.531	
	Antibiotic treatment	16.2 (1.06,4.51)		0.002		Antibiotic treatment	1.2e08 (-5.45,3.2e3)		0.991		Antibiotic treatment	2.947 (-2e3,3,1e3)		0.992		Antibiotic treatment	2.3e7 (-3e3,3e3)		0.992	
	Steroid treatment	2.8 (-0.68,2.75)		0.241		Steroid treatment	7.88 (0.44,3.69)		0.013		Steroid treatment	4.66 (-0.12,3.21)		0.69		Steroid treatment	1.03 (-1.41,5)		0.975	
Covariates	Dysbiosis index	61.1 (-1.1,9.55)		0.122		Dysbiosis index	3.7e32 (0.211,6)		0.042		Dysbiosis index	4.7e5 (2.1,24.1)		0.020		Dysbiosis index	0.39 (-7.5,5.6)		0.767	
	Age	0.9 (-0.04,0.04)		0.999		Age	1.02 (-0.02,0.06)		0.357		Age	0.96 (-0.09,0.16)		0.161		Age	1.07 (0.012,0.12)		0.018	
	Gender	2.1 (-0.47,1.95)		0.229		Gender	1.62 (-0.74,1.7)		0.441		Gender	5.32 (-0.14,3.5)		0.071		Gender	6.71 (-0.264, 07)		0.085	
	Study center	0.45 (-2.03,0.44)		0.211		Study center	1.84 (-0.55,1.78)		0.304		Study center	2.62 (1.14,5.39)		0.003		Study center	1.66 (-0.811, 32)		0.451	
	Antibiotic treatment	15.0 (6 (0.96,4.46)		0.003		Antibiotic treatment	9.9e07 (-2.9e3,-2.9e3)		0.991		Antibiotic treatment	4.7e7 (-4.6e5, 4.6e5)		0.994		Antibiotic treatment	2.0e7 (-3e3,3e3)		0.992	
	Steroid treatment	2.69 (-0.69,2.67)		0.247		Steroid treatment	7.24 (0.35,3.6)		0.017		Steroid treatment	5.12 (-0.13,3.4)		0.069		Steroid treatment	0.93 (-1.621, 29)		0.826	

Next, we aimed to determine whether taxa, microbial genes and metabolic pathways as different features of the oropharyngeal microbiome are significantly associated with ICU admission, need for mechanical ventilation and mortality as major outcomes of hospitalized COVID-19 patients. We aggregated the microbiome and clinical data of patients with moderate and severe SARS-CoV-2 infection, and established a random forest (RF)-based machine learning model (Extended Data Fig. 8). To achieve an independent training and validation of the algorithm, the data were segregated into a training and validation cohorts based on geographic distribution of hospitals (hospitals in the Heidelberg area vs. those outside this region, respectively [see Extended Data Fig. 9 for clinical characteristics after stratification]). Importantly, RF models trained for each of the clinical outcomes in the training set provided significant power to predict these outcomes also in the validation cohort of patients whose data were not included in the training of the RF algorithm (Fig. 3a, Extended Data Fig. 10a, b). Microbiome features were observed to discriminate the need for mechanical ventilation (mean error rate, 0.21; recall, 0.93; area under the receiver operating curve (AUROC), 0.84; Figure 3a), and ICU admission, although to a lesser extent. Incorporation of ICU admission, mechanical ventilation, Shannon index and dysbiosis score as inputs enabled further improvement of the segregation of mortality (mean error rate, 0.27, 0.27; recall, 0.86, 0.71; AUROC, 0.66, 0.67 for ICU admission and mortality, respectively; Fig. 3a).

Given the power of the obtained RF models to discriminate clinical outcomes based purely on the oropharyngeal microbiome, we analyzed the importance of species, genes and pathways in these models and observed partial overlaps of individual microbial features (Fig. 3b; Gini importance > 0.05). As such, *Haemophilus* and *Streptococcus spp.* were among the most important taxa that segregated patients admitted to an ICU and received mechanical ventilation. An acetolactate synthase and pathways involved in DNA and amino acid biosynthesis were among the most important functional features to segregate these two clinical features (Fig. 3c, d; Gini importance > 0.1). Notably, upon stratification into the training and validation cohort, the differences in alpha diversity and dysbiosis between moderate and severe COVID-19 persisted (Extended Data Fig. 10c, d, e). Furthermore, the significant associations of dysbiosis scores with ICU admission and mechanical ventilation were replicated in this setting (Extended Data Fig. 11).

Previously, COVID-19 severity has been linked to several immunological changes such as lymphocytopenia¹⁷, disinhibited releases of proinflammatory cytokines⁶, hyperactivation of monocytes and macrophages¹⁸, dampened type-I interferon (IFN) signaling¹⁹, or a hypercoagulable state²⁰. In a recent case series of eight patients, the lung microbiome in COVID-19 (non-accessible to the majority of treated patients) was reported to be reduced in diversity and enriched with facultative pathogens²¹. Here, we also observed that the readily accessible oropharyngeal microbiome of hospitalized patients with severe COVID-19 was mono-dominated by facultative pathogens with a loss of microbial richness and high dysbiosis. The loss of (beneficial) species, e.g., bifidobacteria or *Prevotella spp.*, in severe cases mirrored the loss of microbial genes and metabolic pathways in these patients. In this context, it is worth noting that the diversity within the oral microbiome has been previously found to be significantly associated with CD4/CD8 T cell ratios and with CD8 T cell activation in HIV-infected patients³¹. Whether such a loss of diversity and, in particular microbial genes, also affects systemic immunity against SARS-CoV2 and thereby facilitates the development of severe COVID-19 still needs to be determined. In general, a mild SARS-CoV-2 infection did not affect microbiome diversity or composition when patients presented during initial coronavirus testing, confirming recent results from an Italian cohort²². As we were not able to follow-up with this mildly affected, community-followed patient subset, potential microbiome contribution to their clinical course merits future studies.

A few machine learning prediction-based models have been attempted in COVID-19 infection, relying on highly variable serum biomarkers, cytokines, blood immune cell counts (representative studies and results are depicted in Extended Data Fig. 12). These analyses revealed AUC/AUROC scores ranging from 0.845 to 0.95 to predict COVID-19 severity or mortality, which are comparable to our AUROC scores, e.g., for mechanical ventilation as a major characteristic of severe COVID-19. Our RF models are purely based on microbiome signatures, but nevertheless these features were suitable to discriminate clinically critical endpoints, severity of the diseases and therapeutic features such as ICU admission, mechanical ventilation and mortality. Due to its cross-sectional design with oropharyngeal microbiome collection close to hospital admission, the predictive power of our observations may be limited to hospitalized COVID-19 patients and also requires independent confirmation.

With these limitations notwithstanding, utilization of the readily accessible oral microbiome signature at admission, as noted in our study, may enable data-driven patient stratification, leading to improved follow-up and tailored management from earlier disease stages, optimization of allocation of critical care supplies and staff, and enhanced ability to safely relocate at-risk patients to centers with maximum care level prior to clinical deterioration.

Methods

Study cohort description

Recruitment. Seven German medical centers participated in the recruitment of inpatient subjects (both sexes, 37-85 years of age) with laboratory confirmed COVID-19 (n=93): University Clinic Heidelberg (Thoraxklinik and Department of Gastroenterology and Infectious Diseases), University Hospital Mannheim, University Clinic Regensburg, University Clinic Frankfurt, Klinikum rechts der Isar of Technical University Munich, and University Heart Center Freiburg. As a control for patients with severe COVID-19 that were admitted to an intensive care unit, we included oropharyngeal biospecimen from 30 SARS-CoV-2 negative patients (both sexes, 29-94 years of age) that were treated at the ICU at the University Clinic Heidelberg because of pulmonary infections and respiratory distress (see Extended Data Fig. 4 for demographic details). Recruitment of outpatient subjects (n=148, both sexes, 18-86 years of age; Extended Data Fig. 1) was performed in COVID-19 test centers run by the Heidelberg public health department. Testing was initiated by health authorities because of emerging, new flu-like symptoms, individual contact with SARS-CoV-2 infected people or travel to COVID-19 outbreak areas. Adult healthy controls (n=74, both sexes, 23-70 years of age; no SARS-CoV-2 infection, no symptoms for upper respiratory tract infections) were recruited among volunteers from DKFZ, University Clinic Heidelberg and the National Center for Tumor Diseases Heidelberg.

Regulatory compliance. The study was approved by the Ethics Committee of the University of Heidelberg (# S-194/2020, S-240/2020 and S-148/2020) according to the principles of the Declaration of Helsinki and is registered at the German Clinical Trials Register (# DRKS00021424). Informed consent was obtained from all the participants of the study.

Participant metadata. Epidemiological, clinical, laboratory, treatment and outcome data of inpatient study participants were extracted from medical records using a standardized version of the WHO/International Severe Acute Respiratory and Emerging Infection Consortium case record form for severe acute respiratory infections⁶. Baseline epidemiologic characteristics, symptoms of infection and antibiotic use were captured from healthy volunteers and outpatient participants.

Assessment of COVID-19 severity according to the WHO guidance document for clinical management of COVID-19 (May 27th, 2020). Symptomatic patients who were tested positive for SARS-CoV-2 infection without evidence of pneumonia or hypoxia were categorized as mild COVID-19. These were largely patients that were screened for the infection in the outpatient test center. Patients admitted to the hospital

with signs of pneumonia but no signs of severe pneumonia (including $\text{SpO}_2 \geq 93\%$ on room air) and tested positive for SARS-CoV-2 were considered to have a moderate disease. Severe COVID-19 was assigned to cases with severe pneumonia (respiratory rate > 30 breaths/min; or $\text{SpO}_2 < 93\%$ on room air), acute respiratory distress syndrome, sepsis or septic shock. For six patients in the severe COVID-19 group that received mechanical ventilation, we do not have gender, age or clinical outcome information. Clinical outcomes were monitored until June 8th, 2020.

Observational study design consideration. This manuscript describes an observational cohort study and was prepared, where applicable, according to the reporting recommendations for observational studies (STROBE Statement)²³. The oropharyngeal specimens were collected prospectively between 30 March 2020 and 20 May 2020 at the different study centers either with the goal of microbiome sequencing and CoV-2 detection, or with the goal of assembling biospecimen banks that would facilitate many different analyses.

Specimen collection and processing

Collection. Oropharyngeal (throat) swabs for microbiome analysis were collected upon testing in an outpatient COVID-19 test center using a DNA-RNA collection kit (Zymo Research, CA, USA). The swabs were collected in addition to diagnostic swabs for laboratory confirmation of SARS-CoV-2 infection. For patients admitted to the hospital oropharyngeal specimen for microbiome analyses were collected primarily at admission day again with the Zymo DNA-RNA collection kit; 15 samples from the University Clinic Regensburg were collected by pharyngeal rinses with sterile water. The rinse solutions were centrifuged, and the pellet was resuspended in an RNA-DNA stabilizing buffer. The oropharyngeal specimens from healthy volunteers were also sampled using the Zymo Research collection kit, and these biospecimens were used for both virus detection and microbiome sequencing.

SARS-CoV-2 RT-PCR. Laboratory confirmation of SARS-CoV-2 for hospitalized patients was performed in certified diagnostic departments of the participating university hospitals. RT-PCR assays were performed in accordance with the protocol established by the WHO / Dr. Drosten (Charité, Universitätsmedizin Berlin, Germany)²⁴ applying an assay kit from Tib-Molbiol (Berlin, Germany) with the E and RdRP genes as RT-PCR targets. The same method was applied to assess the SARS-CoV-2 status of outpatients and healthy volunteers after RNA isolation using the Viral RNA Mini kit (Qiagen, Hilden, Germany).

Metagenome sequencing

For microbiome analyses, DNA was extracted from 200 μL of one aliquot of collected swab material using the QIAamp Fast DNA tissue kit (Qiagen) as per manufacturer protocol. DNA concentrations were measured using a Qubit 4.0 fluorometer with a Qubit™ 1X dsDNA HS Assay-Kit (Life Technologies, Grand Island, NY, USA, Cat. No Q33230). Metagenomes were generated from the resulting DNA, and whole-genome libraries were prepared as follows. DNA was normalized to a concentration of 0,2 ng/ μL . Illumina sequencing libraries were prepared from 1 ng DNA using the Nextera XT DNA Library Preparation kit

(Illumina, San Diego, CA, USA, FC-131-1096) according to the manufacturer's recommended protocol, with reaction volumes scaled accordingly. Concentrations for each pooled library were determined using Qubit™ 1X dsDNA HS Assay-Kit (Life Technologies, Grand Island, NY). Prior to sequencing, libraries were pooled by collecting equal volumes of normalized libraries (2 µl) from batches of 96 samples. The resulting multiplexes were sequenced on Illumina NovaSeq 6000 to obtain 100 bp paired-ends reads with Unique Dual Barcodes to yield ~30-80 million paired end reads per sample. Post-sequencing demultiplexing and generation of FASTQ files was generated using the bcl2fastq Conversion Software (Illumina, San Diego, CA, USA).

Analyses of microbial sequencing data

Metagenomic data processing. Sequencing reads from each sample in a pool were de-multiplexed based on their associated IDT barcode sequence. Next, in order to remove host contaminant reads, sequencing reads mapping to the human genome were first filtered out using KneadData version 0.7.2. Taxonomic profiles of shotgun metagenomes were generated using Kraken 2 version 2.0.8-beta²⁵ followed by re-estimation of species abundance using Bracken version 2.5.3, using the MiniKraken2_v1_8GB kraken database built from the Refseq bacteria, archaea and viral libraries (version of database update: 29/04/23, <https://ccb.jhu.edu/software/kraken2/downloads.shtml>). Functional profiling of metagenomes was performed by HUMAnN2 (<http://huttenhower.sph.harvard.edu/humann2>)²⁶. Here, reads are mapped against a UniRef-based protein sequence catalogue to extract the abundance profiles of genes and gene families (UniRef90). Rarefaction of the metagenome data was done as described below.

Microbiota diversity indices. Alpha-diversity is a mathematical value that summarizes a microbial community according to the count of unique species within a sample and how evenly their frequencies are distributed in the community. Here we calculated alpha-diversity using the Shannon index at the species level that weights also relatively rare abundant species²⁷. To analyze differences in the microbiome composition (i.e., beta-diversity) between samples, we performed a principle coordinates analysis (PCoA) based on species-level Bray-Curtis dissimilarity. These differences were also visualized using the t-distributed Stochastic Neighbor Embedding (t-SNE) algorithm where samples with similar compositions are located relatively close to each other, and clusters of distinct microbiome compositions can be separated¹⁶.

Dysbiosis index. The extent of microbiome dysbiosis in each sample was quantified based on Bray-Curtis distances, following a previously described strategy¹⁵. First, the set of all samples was subjected to principal coordinate analysis (PCoA) using Bray-Curtis distances. Next, the centroid of the healthy control samples subset was derived as the median of their values along all PCoA axes. Then, the dysbiosis score of each sample was calculated as the Euclidian distance between its position in the PCoA space and the healthy control centroid.

Quantification and statistical analysis

Association testing. We assessed differences in relative abundances for species between two different subgroups by Wilcoxon tests and applied multiple hypothesis testing correction by the Benjamini-Hochberg False discovery rate (FDR) method as implemented in SIAMCAT R package²⁸. We also performed a confounder analysis herein computing associations using Fisher's exact tests or Wilcoxon tests (for discrete or continuous variables) between different meta-variables and species abundances and illustrated the variance in relative abundance as explained by different meta-variables. For association analyses, taxonomic input data were filtered using abundance filtering with 10^{-3} as cutoff for minimal abundance and after log-transformation. To associate microbiological taxa with age, gender or antibiotic treatment as clinical cofactors we used a multivariate statistical linear regression analysis by the R package MaAsLin2 with default parameters (abundance filtering with 10^{-3} , min. prevalence 0.1, arcsine-sqrt transformation of relative abundances to normalize microbiome profiles)²⁹. Statistical significance was determined by FDR multiple hypothesis testing correction $p < 0.05$.

Logistic regression. Univariate and multivariate logistic regression analyses were used to predict severity of COVID-19 and clinical outcomes stratified by Shannon alpha diversity or the dysbiosis index of the oropharyngeal microbiome of the COVID-19 inpatient cohort (i.e., patients with moderate and severe COVID-19). Shannon, dysbiosis index and age were used as continuous variables.

Validation and training cohort. The multi-center cohort of hospitalized patients with moderate and severe COVID-19 was divided into a training and validation sub-cohort based on geographic areas. The training set consisted of patients from Heidelberg area (Heidelberg University Clinic, University Hospital Mannheim and Thoraxklinik Heidelberg; $n=15$ moderate, 33 severe cases), and the patients from the other medical centers were grouped into the validation data set (University Clinic Regensburg, University Clinic Frankfurt, Klinikum rechts der Isar of Technical University Munich, and University Heart Center Freiburg; $n=12$ moderate and 33 severe COVID-19 cases). Clinical features of the two cohorts are summarized in Extended Data Fig. 4.

Random forest models. Random forest (RF) analysis was performed using the microbiome and clinical data of patients with moderate and severe SARS-CoV-2 infection. Microbiome features were filtered based on fractional abundance above, excluding taxa ≤ 0.0005 , gene ≤ 0.00005 and pathways ≤ 0.001 relative abundance. Due to the sensitivity of the RF classification to the sampling depth, this was performed after samples were rarefied after removal of human reads (rarefaction of taxa data to 16K Kraken-Bracken counts and genes and pathways to 190K HUMAnN2 gene counts). Following these filtrations, the species, microbial gene and pathway abundance matrices were each renormalized to have a sum fractional abundance of 1 per each sample. Random forest classification was applied using the random Forest R package (<https://cran.r-project.org/web/packages/randomForest/>; A. Liaw, M. Wiener, Classification and regression by random Forest R News, 2/33 (2002), pp. 18-22), embedded in a multi-layer machine learning architecture (Extended Data Fig. 8). Machine learning training and validation were performed on the validation and training cohort as described above (Heidelberg area patients [$n=43$] vs. patients from the other medical centers [$n=33$ patients] including only patients with clinically annotated data and HUMAnN2-derived gene and pathway counts). In the first machine learning layer, RF models

were trained to predict one clinical outcome – either ICU admission, mechanical ventilation or mortality based on one type of microbiome features – i.e. either taxa, gene, or pathways. Hence 9 different combinations of feature type X outcome type were addressed. For each such combination, 100 forests were trained independently, each containing 10000 trees. For each forest, the data was divided randomly and equally to a training set and testing data sets, such that each set contains an equal number of the two possible states of the given outcome. For reproducibility, the randomization seed was set sequentially to a seed value from 1 to 100 for the 100 forests. In the second layer, RF models were trained to predict the ICU and ventilation clinical outcomes using outcome-specific sets of input features, combining together the taxa, gene and pathway features that had Gini importance > 0.01 for the prediction of the given clinical outcome in the first layer, as well as Shannon index and dysbiosis index as additional features. In the third layer, RF models were trained to predict the ICU and ventilation clinical outcomes, using the features that had Gini importance > 0.01 for the prediction of the corresponding clinical outcome in the second layer. For the ventilation outcome, the RF model that was trained at the third layer was taken as the final predictive model. For the ICU and mortality outcomes, an additional, fourth, layer was applied, including as input features for each sample the percentages of trees voting for ventilation-positive and ICU-positive states in the corresponding random forests of the third layer, as well as the Shannon and dysbiosis indices. The two RF models that were trained in this fourth layer were taken as the final predictive models for the ICU and mortality outcomes, correspondingly. The performance of the random forests models were assessed by (i) confusion matrices, enumerating the total number of true-positive, true-negative, false-positive and false-negative predictions, (ii) mean error rates, calculated based on the enumerating confusion matrices as $(\text{false positive} + \text{false negative}) / (\text{true positive} + \text{true negative} + \text{false positive} + \text{false negative})$, (iii) recall, calculated based on the enumerating confusion matrices as $\text{true positive} / (\text{true positive} + \text{false negative})$, (iv) the area under the receiver operating characteristic curve (ROC AUC) using the ROCR R package³⁰, derived from 100 repeats of random subsampling (starting from randomization seed 1) of the patients to enforce equal representation of the positive and negative cases of the given outcome in each subset to be predicted.

Declarations

Data availability

All data is available in the main text or the extended materials. The sequencing data generated during this study are available at European Nucleotide Archive (ENA) [accession code PRJEB41223]. Code used in the present study are available upon request.

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Author contributions

J.d.C., E.Z., T.H., and R.R. contributed equally. U.M. and C.S.-T. contributed equally. J.d.C., T.H., R.R., R.G., B.B.-B., A.K., D.J., E.E., U.M. and C.S.-T. contributed to study concept and design. J.d.C., T.H., R.R., E.Z., S.S., S.H., H.S., M.N., S.K., T. M-E., A.H., Y.K., A.G., M.M., D.D., J.S., P.H., J.E., and C.S.-T. participated in data collection and analysis. J.d.C., D.K., N.C., R.B., F.H., R.S., M.V., A.T., M.E., B.S., H.P., E.E., and U.M. contributed to literature search, writing the manuscript and data interpretation. J.d.C., E.Z., H.S., and C.S.-T. made figures and tables.

Competing interests

The authors declare no conflict of interests.

Additional information

Extended data is available for this paper.

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References

1. Guan, W. *et al.* Clinical Characteristics of Coronavirus Disease 2019 in China. *N. Engl. J. Med.* **382**, 1708–1720 (2020).
2. Banerjee, A. *et al.* Estimating excess 1-year mortality associated with the COVID-19 pandemic according to underlying conditions and age: a population-based cohort study. *Lancet* **395**, 1715–1725 (2020).
3. Verity, R. *et al.* Estimates of the severity of coronavirus disease 2019: a model-based analysis. *Lancet Infect. Dis.* **20**, 669–677 (2020).
4. Onder, G., Rezza, G. & Brusaferro, S. Case-Fatality Rate and Characteristics of Patients Dying in Relation to COVID-19 in Italy. *JAMA - J. Am. Med. Assoc.* **323**, 1775–1776 (2020).
5. Kuderer, N. M. *et al.* Clinical impact of COVID-19 on patients with cancer (CCC19): a cohort study. *Lancet* **395**, 1907–1918 (2020).
6. Zhou, F. *et al.* Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* **395**, 1054–1062 (2020).

7. Zheng, D., Liwinski, T. & Elinav, E. Interaction between microbiota and immunity in health and disease. *Cell Res.* **30**, 492–506 (2020).
8. De Steenhuijsen Piters, W. A. A. *et al.* Nasopharyngeal microbiota, host transcriptome, and disease severity in children with respiratory syncytial virus infection. *Am. J. Respir. Crit. Care Med.* **194**, 1104–1115 (2016).
9. Lee, K. H. *et al.* HHS Public Access. 51–56 (2020) doi:10.1016/j.annepidem.2019.07.013.The.
10. Tsang, T. K. *et al.* Association Between the Respiratory Microbiome and Susceptibility to Influenza Virus Infection. *Clin. Infect. Dis.* **71**, 1195–1203 (2020).
11. Rowe, H. M. *et al.* Direct interactions with influenza promote bacterial adherence during respiratory infections. *Nat. Microbiol.* **4**, 1328–1336 (2019).
12. Fagundes, C. T. *et al.* Transient TLR Activation Restores Inflammatory Response and Ability To Control Pulmonary Bacterial Infection in Germfree Mice. *J. Immunol.* **188**, 1411–1420 (2012).
13. McCullers, J. A. The co-pathogenesis of influenza viruses with bacteria in the lung. *Nat. Rev. Microbiol.* **12**, 252–262 (2014).
14. Bahrani-Mougeot, F. K. *et al.* Molecular analysis of oral and respiratory bacterial species associated with ventilator-associated pneumonia. *J. Clin. Microbiol.* **45**, 1588–1593 (2007).
15. Lloyd-Price, J. *et al.* Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* **569**, 655–662 (2019).
16. Peled, J. U. *et al.* Microbiota as Predictor of Mortality in Allogeneic Hematopoietic-Cell Transplantation. *N. Engl. J. Med.* **382**, 822–834 (2020).
17. Liao, D. *et al.* Haematological characteristics and risk factors in the classification and prognosis evaluation of COVID-19: a retrospective cohort study. *Lancet Haematol.* **7**, e671–e678 (2020).
18. Merad, M. & Martin, J. C. Pathological inflammation in patients with COVID-19: a key role for monocytes and macrophages. *Nat. Rev. Immunol.* **20**, 355–362 (2020).
19. Bost, P. *et al.* Host-Viral Infection Maps Reveal Signatures of Severe COVID-19 Patients. *Cell* **181**, 1475-1488.e12 (2020).
20. Bowles, L. *et al.* Lupus Anticoagulant and Abnormal Coagulation Tests in Patients with Covid-19. *N. Engl. J. Med.* 1–2 (2020) doi:10.1056/nejmc2013656.
21. Shen, Z. *et al.* Genomic diversity of SARS-CoV-2 in COVID-19 patients. 1–27 (2019).
22. De Maio, F. *et al.* Nasopharyngeal Microbiota Profiling of SARS-CoV-2 Infected Patients. *Biol. Proced. Online* **22**, 20–23 (2020).
23. Von Elm, E. *et al.* The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: Guidelines for reporting observational studies. *UroToday Int. J.* **2**, 20–22 (2009).
24. Wölfel, R. *et al.* Virological assessment of hospitalized patients with COVID-2019. *Nature* **581**, 465–469 (2020).

25. Wood, D. E., Lu, J. & Langmead, B. Improved metagenomic analysis with Kraken 2. *Genome Biol.* **20**, 1–13 (2019).
26. Franzosa, E. A. *et al.* Species-level functional profiling of metagenomes and metatranscriptomes. *Nat. Methods* **15**, 962–968 (2018).
27. Hill, T. C. J., Walsh, K. A., Harris, J. A. & Moffett, B. F. Using ecological diversity measures with bacterial communities. *FEMS Microbiol. Ecol.* **43**, 1–11 (2003).
28. Wirbel, J. *et al.* SIAMCAT: user-friendly and versatile machine learning workflows for statistically rigorous microbiome analyses. (2020) doi:10.1101/2020.02.06.931808.
29. Morgan, X. C. *et al.* Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment. *Genome Biol.* **13**, (2012).
30. Sing, T., Sander, O., Beerenwinkel, N. & Lengauer, T. ROCr: Visualizing classifier performance in R. *Bioinformatics* **21**, 3940–3941 (2005).
31. Annavajhala, M.K. *et al.* Oral and gut microbial diversity and immune regulation in patients with HIV on antiretroviral therapy. *mSphere* **5**, e00798-19 (2020).

Figures

Figure 1

Alpha and beta diversity metrics of the oropharyngeal microbiome. a, Alpha diversity displayed as Shannon indices for the oropharyngeal microbiomes sampled from healthy controls (n=74 samples), patients with SARS-CoV-2 negative upper respiratory tract (URT) infections (n=112 specimens), and SARS-CoV-2 positive patients with mild, moderate or severe COVID-19 (n=36, 27, and 66 samples). b, Principal coordinates analyses (PCoA) based on species-level Bray-Curtis dissimilarity for microbiome samples collected from controls and patients. c, Dysbiosis indices defined as per sample as its Euclidian distance to the centroid of the healthy controls' samples in the 5-dimensional PCoA-projection space of the Bray-Curtis dissimilarities. **p<0.01, ***p<0.0001 (Mann-Whitney test).

Figure 2

Taxonomic, gene and microbial pathway differences. a, Hierarchical clustering of the oropharyngeal samples based on their microbiome-composition (taxa-color-coded bar-plot) similarities (dendrogram). Bottom, color-coded bar indicating for each sample the donor category – healthy subjects, patients with non-SARS-CoV-2 URT infections and COVID-19 with different disease severity; in 23 out of 93 samples of moderate and severe COVID-19 single species were observed with a relative abundance > 0.6. b, Relative

abundances of top 20 species within the oropharyngeal microbiome significantly differing between mild vs. severe and moderate vs. severe COVID-19 (Kruskal-Wallis pairwise testing; p-values presented in Suppl. Table 4). c, Volcano-plots presenting fold changes in gene and pathway fractional abundances of the oropharyngeal microbiome of moderate and severe COVID-19 patients stratified according to severity, high vs. low alpha-diversity (Shannon) and low vs. high dysbiosis. d, Heatmaps of fold changes of individual microbial genes (left) and pathways (right) found significantly under-represented in all the 3 compared stratifications in the high severity, low Shannon diversity and high dysbiosis samples. Color-codes indicate the log2 of the ratio (low / high severity, low / high Shannon and high / low dysbiosis) between the means of relative abundances of the two compared groups, confined by assigning -10 to cases having lower values.

Figure 3

Segregation of clinical outcomes in hospitalized patients by random forest (RF) models. a, The performance of the RF models on the validation cohort assessed via error rates, recall, and area under the receiver operating characteristics curve (AUC; median $\pm$ interquartile range, $n = 100$ random sub-sampling for balances representation of each outcome state) for each clinical outcome. b, Venn diagrams indicating the number of commonly selected microbiome features across the different clinical-outcome

RF models. RF-derived median (n = 100 random forests) Gini importance of features having median importance > 0.1, at the species, gene and pathway level for the prediction of c, ICU admission, and d, need for mechanical ventilation.

Supplementary Files

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