Rectal bacteriome and virome signatures and clinical outcomes in community-acquired pneumonia: An exploratory study

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ABSTRACT

Background Bacterial intestinal communities interact with the immune system and may contribute to protection against community-acquired pneumonia (CAP). Intestinal viruses are closely integrated with these bacterial communities, yet the composition and clinical significance of these communities in CAP patients are unknown. The aims of this exploratory study were to characterise the composition of the rectal bacteriome and virome at hospital admission for CAP, and to determine if microbiota signatures correlate with clinical outcomes.

Methods We performed a prospective observational cohort study in CAP patients, admitted to a university or community hospital in the Netherlands between October 2016 and July 2018, and controls. Rectal bacteriome and virome composition were characterised using 16S ribosomal RNA gene sequencing and virus discovery next-generation sequencing, respectively. Unsupervised multi-omics factor analysis was used to assess the co-variation of bacterial and viral communities, which served as primary predictor. The clinical outcomes of interest were the time to clinical stability and the length of hospital stay.

Findings 64 patients and 38 controls were analysed. Rectal bacterial alpha (\(p = 0.015\)) and beta diversity (\(R^2 = 0.123, p = 0.004\)) of CAP patients differed from controls. Bacterial and viral microbiota signatures correlated with the time to clinical stability (hazard ratio 0.43, 95% confidence interval 0.20–0.93, \(p = 0.032\)) and the length of hospital stay (hazard ratio 0.37, 95% confidence interval 0.17–0.81, \(p = 0.012\)), although only the latter remained significant following \(p\)-value adjustment for examining multiple candidate cut-points (\(p = 0.12\) and \(p = 0.046\), respectively).

Interpretation This exploratory study provides preliminary evidence that intestinal bacteriome and virome signatures could be linked with clinical outcomes in CAP. Such exploratory data, when validated in independent cohorts, could inform the development of a microbiota-based diagnostic panel used to predict clinical outcomes in CAP.

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Introduction

Bacterial and viral pneumonia accounts for more deaths than any other infectious disease worldwide and survivors of community-acquired pneumonia (CAP) are at increased risk of long-term
Research in context

Evidence before this study

Preclinical studies have shown that respiratory tract infections are associated with changes in the composition of gut microbiota, which in turn influence the function of the immune system against respiratory pathogens. These findings have yet to be confirmed in humans and their clinical significance in patients with community-acquired pneumonia (CAP) is unknown. We searched PubMed with the terms “(respiratory tract infections[mh] OR pneumonia[tiab]) AND (microbiota [mh] OR microbiome[tiab]) AND (case-control studies[mh] OR prospective[tiab])” for articles published in any language up to March 20, 2021. The search identified 150 results. There were no prospective studies assessing the effects of CAP on the intestinal bacteriome and virome in humans.

Added value of this study

This exploratory study is the first to characterise the rectal bacteriome and virome during CAP in humans. We show that alterations of the intestinal microbiota during CAP not only involve bacteria, but extend to the viral microbiota. Moreover, by using a validated unsupervised matrix factorisation analysis tool, we identified a gut microbiota signature - characterized by a loss of obligately anaerobic bacteria and Lactococcus phages combined with an enrichment of the bacterial genera Staphylococcus and Enterococcus, and their corresponding bacteriophages - which was independently associated with worse clinical outcomes in CAP patients.

Implications of all the available evidence

The intestinal microbiota has an important role in influencing the function of the immune system against respiratory infections. This proof-of-principle study provides preliminary evidence that alterations of these communities are independently associated with clinical outcomes in CAP patients. These exploratory data, when validated in independent cohorts, could inform the development of a microbiota-based diagnostic panel used to predict disease severity and clinical outcomes in hospitalised patients.

In contrast to the increasing interest in the role of the bacterial microbiome during respiratory infections, little is known about the role of other organisms inhabiting the intestine, such as eukaryotic viruses and bacteriophages, jointly called the virome. The relationship between the virome and the host is dynamic and complex, as these micro-organisms have been shown to directly interact with the bacterial microbiome [13,14]. Bacteriophages contribute to the virulence potential of pathogenic bacteria by suppressing antibacterial immunity and possibly preventing clearance of bacterial infection [15]. These interactions between bacteria and bacteriophages might be relevant to commensal intestinal bacteria. Recent studies suggested the importance of intestinal viruses in both intestinal and systemic diseases. For example, decreased viral diversity was associated with the severity of disease of ulcerative colitis, and changes in the virome over time preceded autoimmunity in type I diabetes-susceptible children [16,17]. Despite of the findings linking both the virome and bacteriome to health and disease, the role of these communities during CAP remains unclear.

To characterise the rectal bacterial and viral communities of CAP patients at hospital admission and following discharge, and to determine if microbiota composition at admission correlates with clinical outcomes, we performed an exploratory prospective, observational cohort study in hospitalised CAP patients and controls. The clinical outcomes of interest were time to clinical stability (defined according to the Halm’s criteria) [18], a common outcome measure for pneumonia [19], and length of hospital stay. We hypothesized that the composition of the bacteriome and virome would be disrupted during CAP and that co-occurrence signatures of these disturbances, identified using unsupervised factor analysis [20], could be associated with clinical outcomes.

Methods

Study design and participants

Participants were recruited as part of the ELDER-BIOME study. Details of recruitment and data collection have been previously published [21]. In short, consecutive patients older than 18 years admitted to a university (Amsterdam UMC, location Academic Medical Centre) or community hospital (BovenIJ hospital) in the Netherlands between October 2016 and July 2018, with the clinical suspicion of a community-acquired pneumonia were included, defined as at least one respiratory symptom (new cough or sputum production, chest pain, dyspnea, tachypnea, abnormal lung examination, or respiratory failure) and one systemic symptom (documented fever or hypothersmia, leukocytosis or leukenopna) and had an evident new or progressing infiltrate, consolidation, cavitation, or pleural effusion on chest X-ray or computed tomography scan. Patients exposed to antibiotics within 48 h prior to hospital admission, with the clinical suspicion of an aspiration pneumonia or hospital-associated pneumonia were excluded from study participation. Subjects of comparable age without acute infection, who presented at the outpatient clinic of the Amsterdam UMC, location Academic Medical Centre, served as controls. The ELDER-BIOME study was compiled in accordance with the Declaration of Helsinki and approved by the local institutional review boards (reference: NL57847.018.16). All eligible participants, or their legal representatives, provided written informed consent.

Procedures

Rectal swabs in Universal Transport Medium (Copan, Murrieta, CA, USA) were collected within 24 h of hospital admission and one month thereafter. This timepoint was selected based on previous studies which have shown that the faecal microbiota remains essentially similar within the first 24 h of antibiotic treatment [22,23]. Rectal swabs were used to increase the likelihood of obtaining a sample
within this time window. Rectal swabs at one month following hospi-
total admission were collected at the hospital ward (for inpatients) or
during a follow-up visit at the outpatient clinic (for discharged patients).
Upon completion of sample collection, bacterial microbiota
were characterised by a 16S rRNA gene sequencing targeting the V3-
V4 region and viral microbiota were sequenced by virus discovery
next-generation sequencing (VIDISCA), using a validated virome-
enriched library preparation, both earlier described by our group
[24–27]. Additional details on the 16S rRNA gene sequencing and
VIDISCA are provided in the Supplementary Methods.

Statistical analysis

All analyses were performed using R (version 3.6.0, Vienna, Austria).
To investigate patterns of covariation between rectal bacteria and
viruses during and following CAP hospitalisation, we employed multi-
omics factor analysis (MOFA) [20,25,28]. MOFA captures the principal
sources of variation in a small number of inferred factors or signatures.
The interpretation of these factors is comparable to the interpretation of
the principal components in principal component analysis. In addition,
MOFA calculates a weight for all bacterial and viral features in each fac-
tor. Features with large weights are highly correlated (positively or neg-
atively) with the factor values. Details on the methods for the
assessment of α- and β-diversity, Spearman correlations and the MOFA
model are provided in the Supplementary Methods.

We fitted both a linear plateau model (rcompanion package) and nat-
ural cubic B-splines (splines package) with 2 internal knots (tertile split)
to model the relation between the Factor 1 value and the (log-trans-
formed) values of our clinical outcome of interest. Next, time-to-event
analysis was performed by defining two groups of CAP patients at admi-
sion based on the optimal cut-point of the MOFA factor value and Shan-
non index for the time to clinical stability, determined using maximally
selected rank statistics [29]. Univariable and multivariable Cox propor-
tional hazard models were used to assess potential predictors of clinical
outcomes. The time to clinical stability was one of our clinical outcomes
of interest and is defined according to the Halm’s criteria, which consist
of six clinical parameters (systolic blood pressure ≥90 mmHg, heart rate
≤100 beats/min, respiratory rate ≤24 breaths/min, oxygen saturation
≥90% or arterial oxygen tension ≥60 mmHg, and temperature ≤37.8°C)
[18]. As the dichotomisation of Factor 1 by an optimal cut-point could
result in an overfitted model, as multiple candidate cut-points are exam-
ined, we calculated adjusted p-values using the maxstat package (func-
tion maxstat.test) on Factor 1 values below the plateau. Additional detail
on the time-to-event analysis is available in the Supplementary Methods.

Data were not normally distributed and are therefore presented as
median with interquartile range (IQR). Continuous data were ana-
ysed using a Mann-Whitney U or Kruskal-Wallis test, which are
robust to unequal sample sizes. False-discovery rate was adjusted for
with the Benjamini-Hochberg procedure. The two-tailed level of sig-
nificance between groups was set at (adjusted) p < 0.05.

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used for analyses and took the decision to submit for publication.

Results

Study cohort

64 patients with CAP were enrolled in this study at admission and
followed up after one month, in addition to 38 control subjects.
Patients who were lost to follow up were excluded. The Consolidated
Standards of Reporting Trials flowchart of the study, including details
on participant inclusion, follow up and exclusion of specimens is depicted in Supplementary Fig. 1. Patient demographics and clinical
characteristics are reported in Table 1. Demographic characteristics,
body mass index, prior antibiotic exposure and comorbidities (diabe-
tes, cardiovascular disease, malignancy, gastrointestinal disease and/or
chronic renal disease) were comparable between CAP patients and
controls. However, CAP patients had a higher prevalence of chronic
obstructive pulmonary disease (COPD; p = 0.029).

Rectal bacteriome diversity and composition of patients with CAP
differs from controls

Analysis of the rectal bacterial microbiome showed a significantly
lower bacterial richness (p = 0.000092; Fig. 1A) and α-diversity
(p = 0.0015; Fig. 1B) in CAP patients at admission, as well as after one month
(p < 0.0001 and p = 0.00012; Fig. 1A,B), compared to controls. In
addition, β-diversity of bacterial communities in CAP patients (at
both timepoints) differed significantly from controls (r2 = 0.023,
p = 0.0040 for admission, and r2 = 0.017, p = 0.013 after one month; Fig. 1C).
We found comparable differences in bacterial α- and β-diversity
between CAP patients and controls when we excluded participants
with antibiotic perturbation in the 90 days up to 48 h prior to hospital
admission, and a subgroup analysis of patients and controls without
COPD showed similar results as well (see Supplementary Fig. 2).
However, this does not rule out the possibility of residual confound-
ing by these or other exposures (e.g. immunosuppressed state).

No significant changes in richness (p = 0.076 for CAP patients at
admission compared to controls), α- (p = 0.34) nor β-diversity
(p = 0.63) of viral microbiota were observed (Fig. 1D–F). In depth
analysis of bacterial composition revealed personalised patterns, yet
Firmicutes and Bacteroides were the predominant phyla in all sam-
ples (Fig. 2A). Interestingly, in some CAP patients, especially patients
one month after hospital admission, we observed high relative abun-
dances of potentially pathogenic bacteria, such as Enterococci and
Staphylococcus species (Supplementary Fig. 3).

Viral communities were largely dominated by environmental sin-
gle stranded (ss) RNA viruses, which mostly represent plant viruses
of dietary origin, and bacteriophages of the order Caudovirales, such
as aquatic and anaerobic gut commensals, but lower occurrences of other Firmicutes
species - were posi-
tively correlated with that of its corresponding phage (Benjamini-
Hochberg adjusted p-values < 0.05; Fig. 2C). Furthermore, the rele-
vant abundance of Lactococcus phage was strongly correlated with higher occurrences of Ruminococcaceae (ρ = 0.55, p = 0.0001) and
Lachnospiraceae (ρ = 0.40, p = 0.0001), both families of obligate
anaerobic gut commensals, but lower occurrences of other Firmicutes
(ρ = –0.37, p = 0.0001; Fig. 2C). We thus concluded that rectal bacte-
rial diversity of CAP patients differs from controls, yet no significant
changes in diversity of viral microbiota are observed.

Variation in rectal bacteriome and virome composition between CAP
patients and controls is captured by unsupervised analysis in Factor 1

To further analyse patterns of covariation between bacterial and
viral communities, we used multi-omics factor analysis (MOFA),
which enables the unsupervised integration of these communities.
Unsupervised multi-omics analyses are powerful tools to unmask
potential phenotypes or discover biomarkers that span across biolog-
ical domains. Moreover, MOFA enables concurrent analysis of the
facilitates the unsupervised identification of a limited number of bacterial and viral taxa that drive the variation of the gut microbiome within the cohort.

CAP patients (at both timepoints) had higher values of Factor 1, the major source of variation, than controls ($p<0.001$; Fig. 3A), indicating that Factor 1 captured variation in bacteriome and virome composition associated with CAP. Specifically, Factor 1 represents a signature based on a small number of bacteria and viruses, which is capable of discriminating CAP patients from controls.

Analysis of the weights - which can be interpreted as a measure of importance of certain bacteria and viruses for the value of a factor - revealed that bacteria positively correlated with Factor 1 were Enterococcus and Staphylococcus species. Genera within the family Ruminococcaceae (Ruminococcus, Ruminococcaceae UCG-014, UCG-005, NK4A214) and other obligate anaerobic commensal gut bacteria (Christensenellaceae R7 group, Desulfovibrio) were negatively correlated (Fig. 3B). Viral features that were positively correlated with Factor 1 were Enterobacteriaceae and Staphylococcus phages, while Lactococcus phage was negatively correlated (Fig. 3C). Therefore, these findings indicate that in this cohort, the bacteriome and virome of CAP patients contains higher abundances of Enterococcus species among others, and lower abundances of obligate anaerobic commensal gut bacteria as well as Lactococcus phages compared to controls (Fig. 4A).

**Bacterial and viral microbiota signatures are coupled with clinical outcomes in patients with CAP**

Because Factor 1 represents changes in rectal bacteriome and virome composition between CAP patients and controls (Fig. 3A), we hypothesized that the microbiota signature represented by this factor could serve as a marker for a healthy rectal microbiome and correlate with clinical outcomes. Using a linear plateau model and cubic spline analysis, we found that low Factor 1 value is associated with a shorter length of hospital stay until a threshold of Factor 1 is reached (Supplementary Fig. 4).

Next, CAP patients at admission were divided into two groups based on the optimal cut-point value of Factor 1 for the time to clinical stability (Factor 1 = −1.01), chosen using maximally selected rank statistics [29]. Baseline characteristics and severity scores, except ethnicity ($p = 0.037$) and smoking history ($p = 0.012$), did not differ significantly between these two groups (Supplementary Table 2). We first asked if Factor 1 correlated with the time to clinical stability (defined according to Halm’s criteria) [18]. Using univariable analyses, we found that a low Factor 1 was associated with a shorter time to clinical stability when compared to patients with a high Factor 1 (HR $0.30; 95\%$ confidence interval (CI) $0.20–0.49$; $p = 0.013$; Table 2). These findings indicate that patients with higher abundances of Enterococcus species and lower abundances of obligate anaerobic commensal gut bacteria and Lactococcus phages, were less likely to become clinically stable ($p = 0.032$; Fig. 4B). Demographic characteristics and comorbidities can be potential confounders in clinical studies. When we controlled for several potential confounders (age, sex, ethnicity, prior use of antibiotics, COPD, immunocompromised status and severity of disease by pneumonia severity index (PSI)) [31] in a multivariable analysis, the relationship between a high Factor 1 value and time to clinical stability remained significant (HR $0.24; 95\%$ CI $0.09–0.66$; $p = 0.0055$; Table 2). We next asked if the value of Factor 1 was associated with the length of hospital stay (LOS), our other outcome of interest. Similarly to the time to clinical stability, a low Factor 1 correlated with shorter LOS when compared to CAP patients with a high Factor ($p = 0.0098$; Fig. 4C). The HR for a favourable outcome (discharge) was $0.37$ ($95\%$ CI $0.17–0.81$; $p = 0.012$; Supplementary Table 3) for patients with a high Factor 1. Moreover, the relationship between the Factor 1 value and LOS remained significant in a multivariable analysis ($p = 0.000.043$; Supplementary Table 3).

**Table 1**

Clinical characteristics of the Study Population.

|                         | Patients (n = 64) | Controls (n = 38) | p-value |
|-------------------------|------------------|------------------|---------|
| Age, y, median (IQR)    | 69 (59–87)       | 69 (63–74)       | 0.56    |
| Male sex, n (%)         | 38 (59)          | 22 (58)          | 1.00    |
| Ethnicity, Caucasian, n (%) | 49 (77) | 32 (84)          | 0.91    |
| BMI, kg/m², median (IQR) | 26 (23–28)    | 30 (24–74)       | 0.09    |
| Past smoker, n (%)      | 31 (48)          | 18 (47)          | 0.80    |
| Influenza vaccination, n (%) | 41 (64) | 19 (50)          | 0.15    |
| Prior antibiotic use, n (%) | 10 (16) | 2 (5)            | 0.21    |
| Comorbidities           |                  |                  |         |
| COPD, n (%)             | 18 (28)          | 3 (8)            | 0.029   |
| Immunosuppressed, n (%) | 18 (28)          | 4 (10)           | 0.066   |
| Cardiovascular disease, n (%) | 49 (76) | 27 (71)          | 0.70    |
| Diabetes, n (%)         | 16 (25)          | 4 (10)           | 0.13    |
| Malignancy, n (%)       | 22 (34)          | 6 (15)           | 0.071   |
| Neurological disease, n (%) | 6 (9)   | 0 (0)            | 0.13    |
| Gastrointestinal disease, n (%) | 8 (12)  | 1 (2)            | 0.18    |
| Chronic renal disease, n (%) | 9 (14)  | 2 (5)            | 0.29    |

**Chronic medication**

| NSAIDs, n (%) | 4 (6) | 4 (11) | 0.069 |
| Antihypertensive agents, n (%) | 26 (41) | 23 (61) | 0.082 |
| Proton pump inhibitors, n (%) | 33 (52) | 13 (34) | 0.13   |
| Oral antidiabetics, n (%) | 10 (16) | 3 (3) | 0.086 |
| Laxatives, n (%) | 12 (19) | 2 (5) | 0.11   |
| Microbiology          |       |       |         |
| Streptococcus pneumonia, n (%) | 11 (17) |       |         |
| Haemophilus influenzae, n (%) | 7 (10) |       |         |
| Influenza virus, n (%) | 6 (9) |       |         |
| Other bacterial pathogen, n (%) | 7 (10) |       |         |
| Other viral pathogen, n (%) | 7 (10) |       |         |
| No causative agent found, n (%) | 25 (39) |       |         |
| Severity of disease and outcome |       |       |         |
| PSI class, median (IQR) | 4 (3–4) |       |         |
| qSOFA, median (IQR) | 1 (0–1) |       |         |
| ICU admission, n (%) | 5 (8) |       |         |
| Time to clinical stability, days, median (IQR) | 3 (2–6) |       |         |
| Length of hospital stay, days, median (IQR) | 4 (3–7) |       |         |

**Abbreviations:** IQR, interquartile range; CAP, community-acquired pneumonia; COPD, chronic obstructive pulmonary disease; NSAIDs, nonsteroidal anti-inflammatory drugs; PSI, Pneumonia severity index; qSOFA, quick Sequential Organ Failure Assessment; ICU, intensive care unit.

4 BMI was assessed in 61 patients. 5 Received influenza vaccination in the past year. 6 Exposure to oral or systemic antibiotics between 90 days and 48 h prior to admission. 7 Immunosuppressive disease was defined as clinically suspected or proven immunodeficiency, the use of immunosuppressive therapy or immunomodulating medication in the past 3 months, including chemotherapy, or the use of more than 10 mg prednisone or equivalent each day for the past 3 months. 8 Time to clinical stability was defined according to the Halm’s criteria. 9, 10

bacteriome and virome composition, as opposed to other methods, such as differential relative abundance or α-diversity. To integrate bacterial and viral communities, MOFA identifies the variability in microbiota composition and uses latent factors or signatures (i.e. sources of variation) to capture this variation. MOFA calculates a value for each sample (the factor values), based on the abundance of specific bacterial and viral taxa within that sample. Hence, MOFA
As the dichotomisation of Factor 1 by an optimal cut-point could have resulted in an overfitted model, we calculated adjusted p-values for examining multiple candidate cut points using the maxstat package. When we derived an optimal cut point on Factor 1 below the plateau, we found a statistically significant relation between the Factor 1 value and the length of hospital stay ($p = 0.046$; Fig. 4C). Importantly, the association between the Factor 1 value and the time to clinical stability was not significant ($p = 0.12$; Fig. 4B) following p-value adjustment for examining multiple candidate cut-points, and thus could be consistent with chance.

Of note, when we used MOFA to capture the variability in only bacterial microbiota - without viral microbiota - there was no significant relationship with time to clinical stability ($p = 0.054$; Supplementary Fig. 5). In addition, $\alpha$-diversity – an earlier proposed marker for a healthy gut microbiome [32,33] – was not associated with time to clinical stability ($p = 0.12$; Table 2), nor LOS ($p = 0.16$; Supplementary Table 3). Taken together, the findings of this exploratory study suggest that variation in a small number of rectal bacteria and viruses during CAP might reflect a previously unappreciated marker of clinical outcomes.

**Discussion**

This prospective observational cohort study shows that alterations of rectal microbiota during CAP not only involve bacteria but extend to viral communities. Moreover, by using a validated unsupervised analysis, we found that disrupted gut microbiota profiles of CAP patients at hospital admission are coupled with altered clinical outcomes. To our knowledge, this exploratory study is the first to characterise both the gut bacteriome and virome during CAP in humans, and the first to demonstrate that alterations of these communities might be associated with clinical outcomes in CAP patients.

We and others have demonstrated in mice that both bacterial and viral respiratory infections are associated with a change in the composition of the bacterial gut microbiota, caused by a combination of physiological and environmental influences [34,35]. For example, respiratory infection-induced anorexia is at least partly responsible for microbiota alterations as food-restricted mice displayed reduced amounts of Lachnospiraceae, a family of obligate anaerobic bacteria capable of producing health-promoting short-chain fatty acids [36]. The present study shows that these associations extend to humans, as patients presenting to the hospital with CAP had bacterial gut communities that markedly differed from those in controls. Their bacteriome was characterised by a loss of commensal bacteria, such as genera belonging to the families Lachnospiraceae and Ruminococcaceae, and higher abundances of Enterococcus and Staphylococcus species. The loss of commensal bacteria during illness enables the overgrowth of these facultative aerobic pathogens [7]. The bacteriome remained disturbed one month following hospital admission, indicating that, in line with previous studies [6,24], the impact of hospitalisation and antibiotic exposure on the microbiome is profound and recovery is slow.

In accordance with earlier cohorts, the rectal virome in our study was dominated by dietary plant viruses, crAss phages and other bacteriophages belonging to the order of Caudovirales [37,38]. CrAss phages have recently been linked to Bacteroides species and other Caudovirales are hosted by commensal intestinal bacteria, such as Firmicutes and Bacteroides [37]. Using Spearman correlations, we observed positive associations between the abundance of several bacteria and their corresponding phage. Despite this dependency of viral communities on their bacterial counterparts and a decrease in bacterial $\alpha$-diversity during and following CAP, the diversity of the virome in our study remained unchanged. The changes in the bacteriome during CAP may not be sufficient to alter viral communities as earlier studies reported that the correlation between viral $\alpha$-diversity and bacterial diversity is only mild, and suggested that viral community diversity may not be affected by the use of antibiotics [38,39]. However, the lack of difference in virome composition in this
exploratory study could also be explained by the large heterogeneity and individual differences in viral α-diversity and community compositions, or by the limited sensitivity of VIDISCA next-generation sequencing [27]. Future studies with higher sequencing depth, the possibility to differentiate between integrated and non-integrated phages and a larger sample size are needed to further clarify the role of the intestinal virome in pneumonia.

We were able to define two groups of patients with CAP based on the reduction of obligate anaerobic bacteria, overgrowth of potentially pathogenic bacteria and reduced abundance of Lactococcal phages, which had a statistically significant difference in the time to clinical stability and duration of hospitalisation. However, only the latter remained significant following p-value adjustment for examining multiple candidate cut-points.
Although faecal domination by potentially pathogenic bacteria (such as *Enterococcus* species) and a decrease in obligate anaerobes in critically ill patients has been associated with an increased risk for death and complications [40,41], preclinical studies showed a protective effect of bacterial microbiota against respiratory pathogens [8–10], we are the first to describe that a rectal microbiota signature may predict clinical outcomes during CAP. This signature, comprising a small number of bacteria and viruses, may serve as a proof-of-concept for the possibilities of microbiota-based prediction of clinical outcomes and, when adequately validated in other cohorts, could potentially inform the future development of a standard multiplex PCR panel used to predict disease severity in CAP.

While transkingdom variation was independently associated with clinical outcomes, there was no significant association between the time to clinical stability and bacterial composition or bacterial α-diversity in our study. Therefore, our findings suggest that the combination of viral and bacterial microbiota markers are potentially better predictors for the severity of disease in respiratory tract infections than changes in bacterial composition alone.

Changes in composition of intestinal microbiota during CAP might not solely be a consequence of disease, but certain obligate anaerobic intestinal bacteria possibly influence the severity of infections. These bacteria produce butyrate, a short-chain fatty acid, that influences alveolar macrophages, enhances activity against respiratory pathogens and potentially reduces persistent lung inflammation during...
pneumonia [9,11,12,42]. Moreover, bacteriophage induced lysis of bacteria is expected to result in the release of molecules (such as lipopolysaccharide and peptidoglycan) that serve as microbe-associated molecular patterns which prime the innate immune system and increase activity against bacterial pathogens [43–45]. Therefore, the reduction of anaerobic bacteria capable of producing butyrate combined with altered abundances of several bacteriophages associated with Factor 1 in our study might have resulted in worse outcomes. Although the dichotomisation of Factor 1 could have resulted in an overfitted model, our linear plateau model suggests that a threshold for a healthy microbiota exists and the loss of a critical amount of beneficial intestinal micro-organisms might be associated with worse clinical outcomes. Of note, the association between the Factor1 value and LOS remained significant following p-value adjustment for examining multiple candidate cut-points, whereas the association between the Factor 1 value and the time to clinical stability was not significant following these adjustments.

Future research should elucidate the exact mechanism by which alterations in intestinal microbiota composition, both bacterial and viral, affect the immune system and influence clinical outcomes. Such studies could potentially lead to interventions such as restoration of an altered microbiota or the use of microbiota-derived metabolites to improve outcomes for CAP patients.

This study was designed as a small proof-of-concept study of exploratory nature and has several limitations. First, despite our efforts to address for confounders when comparing CAP patients with controls (an age-matched cohort with comparable demographic characteristics, antibiotic exposure and comorbidities), the described differences might reflect undocumented differences between these groups (e.g. diet and genetic makeup of the host). Moreover, the observational nature of this study and limited sample size limits the possibilities to control for all possible confounders such as comorbidities and medication use.

Second, although we used a validated approach for viral sequencing, the sensitivity of VIDISCA next-generation sequencing reaches 1 E6 viral genome copies/ml and therefore we might not have identified low abundant viruses, and underestimated inter-individual differences in virome α-diversity and composition which could have been identified by other techniques such as total transcriptome sequencing [27,46]. In addition, the analytical approach of VIDISCA did not allow for the discrimination between integrated and non-integrated bacteriophages. Third, we observed that a rectal microbiota signature (Factor 1) was independently associated with clinical outcomes independent of clinical confounders. However, due to the dichotomisation of Factor 1 and the relatively limited sample size these analyses might have been overfitted, which is underscored by the absence of a significant relation between the Factor 1 value and the time to clinical stability following p-value adjustment for examining multiple candidate cut-points. Fourth, the Factor 1 value is based on a cohort-specific matrix factorization analysis and therefore not generalizable to other patient populations. Consequently, the microbial signatures we identified were not replicated in an independent cohort and warrant confirmation in larger and more diverse patient cohorts, before they can be used to improve triage and patient care in hospital settings.

Finally, although our findings remained significant when controlled for several important confounders, the variation in virome and bacteriome composition at hospital admission for CAP might not influence outcomes, but reflect undocumented differences between CAP patients that lead to worse clinical outcomes.

In conclusion, these results indicate that the combined variation in rectal bacteriome and virome composition could be associated with clinical outcomes in patients with CAP. This exploratory study serves as a proof-of-principle and may inform the future development of a microbiota-based panel used to predict disease severity in CAP.

Declaration of Competing Interest

All authors report no competing interests.

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Author contributions

FH, BWH and WJJW conceived and supervised the study. RKF and BWH verified and analysed the data, wrote the original manuscript and prepared the final figures. XB and BWH conducted data
collection. FH oversaw 16S rRNA sequencing and analysis. CMK, MD, MK and LvdH designed and performed the viral sequencing and bioinformatics pipeline. HPS and JMB oversaw statistical analysis. DRF was the treating physician of included patients and assisted in the manuscript preparation. XB, BPS, TP, BWH and WJW drafted the study design and data collection protocol, and finalised manuscript preparation. All authors critically reviewed and approved the final manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.eclinm.2021.101074.

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