






BMJ Open Early diagnostic BioMARKers in exacerbations of chronic obstructive pulmonary disease: protocol of the exploratory, prospective, longitudinal, single-centre, observational MARKED study

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ABSTRACT

Introduction Acute exacerbations of chronic obstructive pulmonary disease (AECOPD) play a pivotal role in the burden and progressive course of chronic obstructive pulmonary disease (COPD). As such, disease management is predominantly based on the prevention of these episodes of acute worsening of respiratory symptoms. However, to date, personalised prediction and early and accurate diagnosis of AECOPD remain unsuccessful. Therefore, the current study was designed to explore which frequently measured biomarkers can predict an AECOPD and/or respiratory infection in patients with COPD. Moreover, the study aims to increase our understanding of the heterogeneity of AECOPD as well as the role of microbial composition and host-microbiome interactions to elucidate new disease biology in COPD.

Methods and analysis The ‘Early diagnostic BioMARKers in Exacerbations of COPD’ study is an exploratory, prospective, longitudinal, single-centre, observational study with 8-week follow-up enrolling up to 150 patients with COPD admitted to inpatient pulmonary rehabilitation at Ciro (Horn, the Netherlands). Respiratory symptoms, vitals, spirometry and nasopharyngeal, venous blood, spontaneous sputum and stool samples will be frequently collected for exploratory biomarker analysis, longitudinal characterisation of AECOPD (ie, clinical, functional and microbial) and to identify host-microbiome interactions. Genomic sequencing will be performed to identify mutations associated with increased risk of AECOPD and microbial infections. Predictors of time-to-first AECOPD will be modelled using Cox proportional hazards’ regression. Multiomic analyses will provide a novel integration tool to generate predictive models and testable hypotheses about disease causation and predictors of disease progression.

Ethics and dissemination This protocol was approved by the Medical Research Ethics Committees United (MEC-U), Nieuwegein, the Netherlands (NL71364.100.19).

Trial registration number NCT05315674.

STRENGTHS AND LIMITATIONS OF THIS STUDY

- ⇒ The current exploratory, prospective, single-centre, longitudinal, observational ‘Early diagnostic BioMARKers in Exacerbations of COPD’ study was designed to assess early predictors of exacerbations and/or respiratory infection from a panel of frequently measured biomarkers (ie, symptoms, vitals, spirometry and nasopharyngeal, venous blood, spontaneous sputum and stool samples), microbial composition and host-microbiome interactions in patients with chronic obstructive pulmonary disease (COPD) who are referred to an 8-week inpatient pulmonary rehabilitation programme.
- ⇒ The living-lab environment of the pulmonary rehabilitation centre provides a unique setting to comprehensively and longitudinally characterise these events and markers in patients with COPD in the stable state, during exacerbation episodes and during recovery.
- ⇒ This study is the first to use an inpatient setting to explore multiple biological sample types which will provide valuable information on the intravariability and intervariability of biomarkers, microbial composition and host-microbiome interactions within and across individual patients over time.
- ⇒ Despite the intensive sampling and patient monitoring intended to capture exacerbations, other unknown predictors not assessed in the study may be missed.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a common, preventable and treatable disease that is characterised by airflow limitation and respiratory symptoms.¹ Relief of symptoms and prevention of acute

exacerbations of COPD (AECOPD) are important goals in the management of COPD.¹ Although traditionally defined as episodes of acute worsening of respiratory symptoms that require additional pharmacological therapy,¹ an updated definition and severity classification of AECOPD was recently proposed integrating a specified time span with objectively measured and readily available clinical measures.² Exacerbations are common: up to 70% of patients has experienced ≥ 1 AECOPD during a ≥ 1 -year follow-up in multiple large cohort studies.³ However, AECOPD recognition and reporting by patients is generally poor,⁴ contributing to an underestimation of its actual incidence. Exacerbations can have a significant negative impact on lung function,⁵ quality of life,⁶ physical activity,⁷ hospitalisation⁸ and mortality rates.⁹ Moreover, AECOPD explains the majority of COPD-related direct (treatment related) and indirect (productivity related) healthcare costs.^{10 11}

Despite their frequent occurrence and contribution to the personal and societal burden of COPD, adequate prediction and early diagnosis of AECOPD currently remain challenging. In addition to its symptom-based and event-driven definition, lack of predictive and diagnostic biomarkers adds to this challenge. A biomarker is defined as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention and may be used as a diagnostic and/or disease staging tool, as an indicator of disease prognosis or for the prediction and monitoring of specific responses'.¹² Although commonly identified in blood and other tissues, by definition, biomarkers may also comprise clinical parameters and imaging results. The implementation of biomarkers for the early diagnosis of AECOPD, such as circulating C reactive protein and procalcitonin, thus far, has been precluded by a lack of sensitivity, specificity and predictive value. Moreover, inconsistent results and a lack of validation have impeded their clinical applicability to date.^{13–21} While early and accurate biomarkers remain unidentified, the strongest predictor remains a history of AECOPD.²² However, year-to-year variations limit the utility of this predictor in individual patients.^{23 24} To improve the accuracy of predictions, timely diagnosis and subsequent personalised treatment of these events, it is essential to identify early AECOPD biomarkers.²⁵

The James Lind Alliance recently surveyed patients, caregivers and clinicians to collect and prioritise research questions regarding AECOPD.²⁶ The highest rated research question was to identify better methods to prevent AECOPD. One method might be to modulate the microbiome. Indeed, alterations of the respiratory and intestinal microbiota, and host–microbiome interactions, are increasingly recognised for their role in (AE) COPD.^{27–35} A growing body of research has already led to the identification of different biological AECOPD clusters,^{32 36 37} which are linked to distinct microbial profiles.^{36 38–43} Moreover, studies have shown that antibiotics are promising in reducing AECOPD through

bacterial decolonisation, and that sputum microbial composition may be useful in guiding antibiotic stewardship.^{44 45} These findings could strengthen the hypothesis that microbiome modulation may be a potential method to prevent AECOPD.

The 'Early diagnostic BioMARKers in Exacerbations of COPD' (MARKED) study was designed to longitudinally explore predictive AECOPD biomarkers, microbial composition and host–microbiome interactions in patients with COPD. This manuscript describes the objectives, protocol, clinical implications, possible strengths and weaknesses of the MARKED study.

Study objectives

The primary objective of the MARKED study is to explore which biomarkers from a panel of frequently measured biomarkers (symptoms, vitals, spirometry parameters and nasopharyngeal, venous blood, spontaneous sputum and stool samples) predict an AECOPD and/or respiratory infection in patients with COPD, to enable selection of biomarkers for validation in future studies.

Furthermore, secondary objectives are to:

- ▶ Investigate longitudinal alterations in microbial composition and host–microbiome interactions in the stable state, at AECOPD and during recovery.
- ▶ Study the heterogeneity of AECOPD by comprehensive (ie, clinical, functional, microbial, proteomic, transcriptomic, genetic, metabolomic, inflammatory and biochemical) characterisation of these events.
- ▶ Determine the correlation between microbial alterations in the airways/gut and inflammatory biomarkers in blood during longitudinal follow-up.
- ▶ Longitudinally investigate biomarkers of AECOPD in clinically relevant subgroups of patients with COPD (eg, current vs ex-smokers, high vs low blood eosinophils, frequent vs infrequent exacerbators).
- ▶ Comprehensively investigate whether host–microbiome interactions, biomarkers and predictive models identify those patients who do not exacerbate despite having a respiratory infection.

METHODS AND ANALYSIS

The MARKED study is a collaboration between Ciro (Horn, the Netherlands) and AstraZeneca (Mölndal, Sweden and Gaithersburg, USA). Ciro is a tertiary care centre with expertise in the integrated diagnosis and treatment of patients with chronic respiratory diseases. AstraZeneca is a biopharmaceutical company with expertise in research and development of therapeutics.

Patient and public involvement statement

Patients or the public were not involved in the design, conduct, reporting or dissemination plans of the current research.

Study outline

This exploratory, prospective, single-centre, longitudinal, observational study will be enrolling patients with clinically stable COPD who are referred to an 8-week

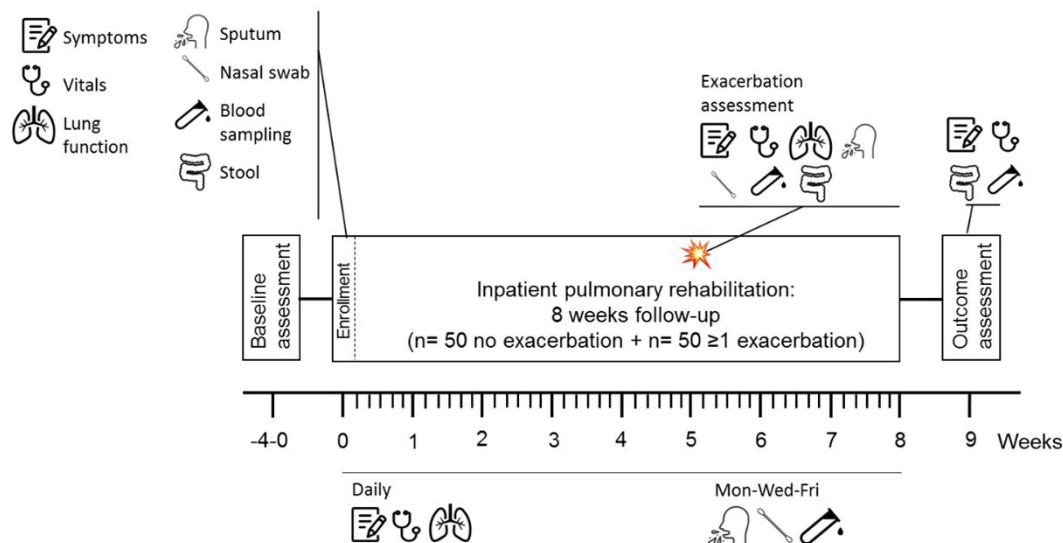


Figure 1 Study design including the timing of study specific measurements. *Indicates exacerbations.

inpatient pulmonary rehabilitation (PR) programme at Ciro. Clinical stability will be defined by haemodynamic and respiratory stability, without initiation of antibiotics or prednisone treatment ≤ 7 days prior to study entry. The expected study duration is 1.5 years from enrolment of the first patient (July 2022) to the last patient (January 2024). To explore early AECOPD biomarkers, a panel of prespecified biomarkers will be assessed on a daily or three times weekly basis, with additional measurements during AECOPD and at outcome assessment (figure 1).

Study population

While aiming to include at least 50 patients experiencing ≥ 1 AECOPD and at least 50 patients without an AECOPD, up to 150 consecutive patients with a primary diagnosis of COPD admitted to the inpatient PR programme in Ciro will be recruited for the study after completing their baseline assessment. Please see the sample size calculation further below for further details.

Inclusion criteria

Patients must meet the following criteria to be eligible for participation in this study:

- ▶ ≥ 40 years old.
- ▶ ≥ 10 pack years of smoking.
- ▶ Primary diagnosis of COPD and postbronchodilator ratio of forced expiratory volume in the first second (FEV_1) to forced vital capacity (FVC) of less than 0.70.¹
- ▶ Clinical indication for inpatient PR at Ciro.
- ▶ Provided written informed consent.

Exclusion criteria

Patients will be excluded from participation in the study if any of the following criteria are met:

- ▶ Current, that is, < 12 months (secondary) diagnosis of asthma according to the referring physician.
- ▶ Unstable concurrent cardiovascular, metabolic, renal, gastrointestinal and musculoskeletal chronic diseases, as judged by the investigator.

- ▶ Chronic use of oral corticosteroids > 10 mg prednisolone/day.
- ▶ Initiation of maintenance therapy with macrolides < 6 weeks prior to study entry.
- ▶ Anaemia, defined as haemoglobin level < 8.1 mmol/L in men and < 7.5 mmol/L in women.⁴⁶
- ▶ Participation in a study involving investigational or marketed products concomitantly or < 8 weeks prior to study entry.
- ▶ Unable to read, speak or understand Dutch.

Recruitment

Patients admitted for a baseline prerehabilitation assessment at Ciro will be informed about the study by the treating physician. The study's patient information form will be provided to all eligible patients. Patients will be contacted by telephone 1 week after the baseline assessment to discuss study participation. If the patient agrees to participate, written informed consent will be obtained at the first day of the PR programme, which marks study enrolment. The date of study enrolment as well as of the entire follow-up period will be documented to be able to study and correct for potential seasonal variations.

Exacerbation diagnosis and classification

Exacerbation-like events are evaluated by the chest physician in Ciro within 24 hours of onset. AECOPD are defined by an increase in respiratory symptoms according to the criteria of Anthonisen,⁴⁷ and the need for additional pharmacological treatment.¹ Routine clinical tests are performed including the assessment of dyspnoea, oxygen saturation, respiratory rate, heart rate, C reactive protein and arterial blood gases.² Furthermore, differential diagnoses such as pneumonia, pneumothorax or acute heart failure are ruled out. Therefore, chest X-ray and a 12-lead ECG are performed. Venous blood is collected for routine laboratory analyses of specific AECOPD markers. Furthermore, spontaneous sputum is collected (if produced) and

analysed for routine traditional bacterial culture. Arterial blood is collected from the radial artery to determine arterial blood gases when acute respiratory failure is clinically suspected.

All AECOPD evaluated as moderate or severe by the physician in Ciro will be recorded in this study. Moderate AECOPD are defined by a worsening of symptoms >2 consecutive days leading to treatment with systemic glucocorticoids, antibiotics or both. Severe events are defined as moderate AECOPD necessitating treatment with (enhanced) oxygen therapy, intensification of long-term home non-invasive ventilation (NIV) or escalation to hospital care facilities >24 hours for care not available in Ciro (eg, intravenous treatment and mechanical ventilation).

Outcomes

Measurements collected as part of routine clinical care as well as study-specific measurements will be integrated. A complete overview is provided in online supplemental table 1.

Primary outcome

Main study parameters include the daily assessment of respiratory symptoms, vital signs and pre-bronchodilator spirometry. The validated EXAcerbations of Chronic pulmonary disease Tool⁴⁸ and COPD-Lower Respiratory Tract Infection-Visual Analogue Score⁴⁹ will be administered for the standardised assessment of respiratory symptoms. Vital signs will be recorded at rest and include the assessment of systolic and diastolic blood pressure, heart rate, oxygen saturation, body temperature and breathing frequency. Prebronchodilator spirometry will be performed by certified and experienced respiratory technicians using Vyntus One (Vyair Medical, Würzburg, Germany) to record the FEV₁, FVC, FEV₁/FVC ratio and peak expiratory flow.

Main study endpoints furthermore include the collection of nasopharyngeal swabs, venous blood, spontaneously produced sputum and stool for extensive microbial, metabolomic, proteomic, transcriptomic and inflammatory characterisation. Nasopharyngeal swabs will be performed on both nostrils and are processed and stored at -20°C. All other biospecimens will be processed and stored at -80°C at Ciro until batch shipment to the AstraZeneca biorepository (Gothenburg, Sweden) for further laboratory analyses. Please see [table 1](#) for a detailed overview of the corresponding analyses.

Some time considerations should be noted. First, all biological samples will be collected in the morning. To ensure a successful collection of the not on-demand samples (ie, spontaneous sputum and stool), these samples may be collected within 24–48 hours after study enrolment. Similarly, the collection of stool at outcome assessment will be performed in the last week of study follow-up. Furthermore, all exacerbation assessments will be performed within 72 hours of onset of the event.

Secondary outcomes

Basic characteristics

Secondary study parameters include patient characteristics, including: age, sex, race, marital status, current smoking status and smoking history, medical history, current medication use, oxygen and NIV use as well as AECOPD and hospitalisations in the 12 months prior to admission.

Comorbidities

Self-reported and physician-reported comorbidities will be collected at study enrolment. As part of routine clinical care, an ECG is performed to objectively identify arrhythmia and previous myocardial infarction.⁵⁰ Furthermore, systolic and diastolic blood pressure are measured to diagnose arterial hypertension.

Lung function and respiratory muscle strength

Besides the daily prebronchodilator spirometry tests, postbronchodilator spirometry, body plethysmography, diffusing capacity and respiratory muscle strength will be recorded at baseline and outcome assessment as part of routine clinical care. As such, total lung capacity, residual volume, intrathoracic gas volume, diffusing capacity for carbon monoxide, Krogh's diffusion constant and maximal static inspiratory and expiratory mouth pressures will be captured.

Chest HRCT

If available from the previous 12 months, a chest high-resolution CT (HRCT) scan will be collected at study enrolment for *post hoc* radiological quantification of the pulmonary and extrapulmonary features of COPD.

Body composition

Body composition will be recorded at baseline assessment as part of routine clinical care. Body length and weight are measured to the nearest 0.5 cm and 0.1 kg, respectively, to calculate the body mass index (weight/length²). Body composition is assessed by Dual-Energy X-ray Absorptiometry (DEXA; Lunar iDXA, GE Healthcare—enCORE V.14, Madison, Wisconsin) to record bone mineral density, fat-free mass (FFM) and the fat-free mass index (FFMI (FFM/length²)).

Patient-reported outcomes

Self-reported dyspnoea is scored using the modified Medical Research Council questionnaire (scale 0–4).⁵¹ Health status will be assessed using the COPD Assessment Test (CAT).⁵² The CAT consists of 8 items and provides a total score ranging from 0 to 40 points. Furthermore, symptoms of anxiety and depression will be assessed using the hospital anxiety and depression scale (HADS).⁵³ The HADS is divided into an anxiety subscale and a depression subscale, with total scores ranging from 0 to 21 in each subscale. These measures will be collected at baseline and outcome assessment, as part of routine clinical care.

Table 1 Overview of sample collections and analytical purposes

Sample	Type	Timing	Analysis	Readouts
Nasopharynx	eNAT (Copan)	Enrollment, 3x weekly, exacerbation	Amplicon sequencing (16S rRNA and ITS)	Diversity estimation and relative abundances of bacterial (bacteriome) and fungal (mycobiome) taxa
			WMTS	Identification of RNA viruses and host transcripts
Venous blood	PAXgene DNA (BD)	Enrolment	Genomic sequencing	SNP identification associated with increased risk of COPD, exacerbations and microbial infection
	PPT (BD)	Enrolment, exacerbation, outcome assessment	WMTS	Plasma derived ECV assessment to identify circulating host transcripts
	Cell-free DNA (Streck)		WMTS	Assessment of circulating microbial DNA
	SST (BD)	Enrollment, 3x weekly, exacerbation, outcome assessment	ELISA-based multiplex analysis	Assessment of broad multiplex cytokine panel
			MSD	Anti-bacterial titers
			Metabolomics	Host and microbiome derived metabolites
			Proteomics	Host protein characterisation
PAXgene RNA (BD)		WMTS	RNA virus and host transcript identification	
Spontaneous sputum	N.A.	Enrollment, 3x weekly, exacerbation	Amplicon sequencing (16S rRNA and ITS and qPCR for NTHi)	Diversity estimation and relative abundances of bacterial (bacteriome) and fungal (mycobiome) taxa
			WMTS	RNA virus and host transcript identification
			Proteomics	Host protein characterisation
			Metabolomics	Host and microbiome derived metabolites
		Enrollment, exacerbation	Traditional bacterial culture*	Bacterial taxa identification
			Cytospin	Cell differentials
Stool	OMNIgene Gut	Enrolment, exacerbation, outcome assessment	Whole metagenomic shotgun sequencing	Microbial taxa characterisation at species level, and functional profiling
	OMNImet Gut		Metabolomics	Host and microbiome derived metabolites

*Supporting both routine clinical care and study specific measurements at exacerbation.

BD, Becton Dickinson; ECV, extracellular vesicle; ELISA, enzyme-linked immunosorbent assay; ITS, internal transcribed spacer; MSD, Meso Scale Discovery Electrochemiluminescence; NTHi, *Nontypeable Haemophilus influenzae*; qPCR, quantitative PCR; rRNA, ribosomal ribonucleic acid; SNP, single nucleotide polymorphism; WMTS, whole metatranscriptomic sequencing.

Exercise capacity

Exercise capacity will be assessed using cardiopulmonary exercise testing (CPET),⁵⁴ a constant work rate cycle test (CWRT)⁵⁵ and the 6 min walk test (6MWT)⁵⁶ at baseline and outcome assessment, as part of routine clinical care. CPET will be performed using the Vyntus CPX cycle ergometer (Vyaire Medical, Würzburg, Germany) to assess maximal exercise capacity (W_{max}), supervised by a physician. Submaximal exercise capacity will be assessed by CWRT at a workload of 75% of the predetermined W_{max} (± 2 Watt) on the same cycle ergometer, supervised by certified and experienced technicians. Furthermore, 6MWT will be performed two times, on separate days, in line with international recommendations.⁵⁶

Muscle function

Muscle function measurements will be performed at baseline and outcome assessment, as part of routine clinical care. Isometric and isokinetic quadriceps strength will be assessed using a computerised dynamometer (Biodex System 4 Pro, Biodex Medical Systems, New York). Isometric quadriceps strength will be defined by the highest peak torque, and isokinetic quadriceps strength will be defined by the total amount of delivered work.⁵⁷

Laboratory analyses

Venous blood will be collected for routine haematology and chemistry at baseline and outcome assessment. The total concentration of haemoglobin, hematocrit, thrombocytes, leukocytes including differentiation (absolute and

relative), sodium, potassium, urea, creatinine, aspartate aminotransferase, alanine aminotransferase, bilirubin, cholesterol, high-density lipoprotein, low-density lipoprotein, triglycerides, high sensitive C reactive protein) and glycated haemoglobin are determined. Furthermore, an arterial blood sample is collected at baseline assessment to determine the resting arterial partial pressure of oxygen (P_aO_2), carbon dioxide (P_aCO_2) and oxygen saturation. Patients with long-term oxygen therapy will continue oxygen supply during the procedure.

Safety reporting

In accordance with the Medical Research Involving Human Subjects Act (WMO) the study will be suspended if the health or safety of subjects will be jeopardised. The accredited Medical Ethical Teaching Committee (Medical Research Ethics Committees United (MEC-U), Nieuwegein, the Netherlands) will be notified without undue delay after obtaining knowledge of these events. Adverse and serious adverse events will be recorded (hospitalised). AECOPD will not be considered as serious adverse events: exacerbations and hospitalised AECOPD will be annually reported to MEC-U. Ciro has established protocols for the management of AECOPD: these protocols will also be followed for patients included in the study. Moderate AECOPD are treated at Ciro, the PR programme will be adjusted as needed. Patients with severe AECOPD requiring hospital admission will be referred to a nearby hospital.

Sample size calculation

Although sample size rules of thumb exist for multivariable regression modelling and prediction modelling strategies, this study is explorative in nature. Therefore, the amount of biomarkers exceeds the amount when applying the rule of thumb that states that for each potential predictive variable, 10 events should be observed. The study aims to include at least 50 unique individual patients who experience ≥ 1 AECOPD, and at least 50 individual patients without an AECOPD, to provide accurate benchmark data (ie, sufficient precision to estimate mean and SD) for exploratory biomarkers. From previous studies in Ciro, it is known that approximately 42% of patients will develop at least one AECOPD during admission.⁵⁸ Because AECOPD are unpredictable and variable, which is an important rationale for the present study, the study will expectedly need 100–150 patients to be included. The goal of this study is not to develop a multivariate model, but rather to explore the associations between biomarkers and the occurrence of an event.

Data management and statistical analyses

In case of missing data on predictors, stochastic regression imputation with fully conditional specification will be used to impute the data set to allow the use of all included patients for the analyses. Values will be drawn using predictive mean matching. Baseline characteristics will be reported as mean and SD or as median and IQR

for continuous variables, as appropriate, and as count and percentage for categorical characteristics. Predictors of time-to-first AECOPD during the study period will be modelled using univariate and multivariable Cox proportional hazards regression. Associations will be presented as HR and 95% CI. The dependency of the predictive performance of biomarkers will be tested using interaction terms. The concordance-statistic, or c-statistic, will be estimated to assess discriminative ability. Time-dependent area under the receiver operating characteristic plots will furthermore be created. Calibration will be assessed by comparing the predicted probability with the observed probability of an AECOPD and examined with a calibration plot and calibration slope, assuming no data censoring before the end of follow-up.

Characterisation of the microbiome will be determined by alpha diversity and beta diversity and relative abundance of bacterial taxa. Alpha diversity will be treated as a continuous variable and analysed using appropriate statistical tests, whereas ordination of beta-diversity distances will be done using principal component analysis. Multiomic analyses will be used to generate hypotheses about the drivers that promote progression to AECOPD. Differential enrichment analysis for feature selection across all 'omics will be done after accounting for multiple hypothesis testing using a robust model that considers distributional assumptions. The association between genetic variants and (AE)COPD and microbial infections will be studied using whole-exome sequencing data.

Self-organising maps (SOMs, also referred to as Kohonen maps) will be used to create an ordered representation of the selected attributes at the time of AECOPD by using Viscovery Profiler V.7.1 (Viscovery Software GmbH, Vienna, Austria). Based on the identified homogeneous data groups created in the SOM model, clusters will be generated using Viscovery's SOM-Ward Cluster algorithm. Summary variables of clinical characteristics for the total sample, and for clusters, will be presented as mean and SD for quantitative variables, and as percentages for discrete variables. Differences between groups will be assessed using integrated two-sided t tests. Repeated measure correlations will be used to determine the within-individual association between microbial alterations in the airways/gut and systemic inflammatory biomarkers across patients.

Statistical significance will be denoted by $p < 0.05$.

Ethics and dissemination

Ethical approval for the study has been granted by MEC-U, Nieuwegein, the Netherlands (NL71364.100.19). The MARKED study is registered at clinical trials. The study will be conducted according to the principles of the Declaration of Helsinki (64th WMA General Assembly, Fortaleza, Brazil, October 2013⁵⁹), and in accordance with the Good Clinical Practice WMO guidelines. Written informed consent will be obtained from all participants prior to study participation. Results of the study will be

published in peer-reviewed scientific journals and will be presented at (inter)national conferences. If desired, participants will be informed about the outcomes of the study.

Discussion

The results of the MARKED study will increase our understanding of early AECOPD biomarkers, including associations with microbial characteristics, longitudinal alterations in microbial composition and host–microbiome interactions in patients with COPD. This knowledge is essential for the development of novel anti-microbial and other therapeutic targets to prevent unnecessary treatment and healthcare utilisation, to improve the management and personalised treatment of (the different types of) AECOPD, to reduce the future impact of AECOPD and, thus, to improve overall care for individual patients. Since AECOPD are heterogeneous events in terms of origin, trigger, severity, duration, need for treatment and overall clinical presentation,^{1 22 27 37 60–63} it is anticipated that complex biomarker panels, rather than a single biomarker, will be identified for the different subtypes of AECOPD. Moreover, AECOPD diagnosis relies heavily on the exclusion of differential diagnoses,¹ which further decreases the potential of a single predictive AECOPD biomarker.

Major strengths of the MARKED study are the comprehensive and longitudinal characterisation of clinical, laboratory and microbial variables associated with AECOPD. This will enable us to increase our understanding of explorative biomarkers as well as the heterogeneity of AECOPD in general. The living-lab environment of the PR centre and the study's prospective longitudinal design provide a unique setting to study such markers. Indeed, only a few studies have focused on the time window between AECOPD trigger and onset of deteriorating symptoms.^{64 65} In addition, the inclusion of multiple types of biological samples will provide relevant information on the intravariability and intervariability of biomarkers and, thus, their clinical potential. To our knowledge, no studies have yet explored the longitudinal alterations in microbial composition and host–microbiome interactions in different types of biological samples within and across individual patients. Furthermore, no serious risks are associated with study participation. Participants are even likely to benefit from close monitoring and early initiation of treatment, as indicated.

We also note several possible limitations. First, the study population will consist of a convenience sample enrolling patients with COPD admitted for inpatient PR. While PR is indicated for all grades of symptomatic COPD,^{66 67} it is particularly indicated in patients with moderate to severe disease.^{1 68} As a result, this study population may be biased towards the most vulnerable patients, compromising the study's generalisability to all patients with COPD. Nevertheless, patients with a primary diagnosis of COPD independent of disease severity are eligible for inclusion. Therefore, selection bias is minimised, at least to a certain

extent. A time span of 72 hours within onset will be applied to increase the chances for successful sample collections during AECOPD. Nevertheless, some assessments may be missed during severe AECOPD due to acute hospital admission. In addition, the sampling frequency may be considered as burdensome and might contribute to non-participation and drop-out. While mild-to-moderate AECOPD usually do not affect drop-out rates, research has pointed out that particularly severe AECOPD are associated with drop-out of PR.⁵⁸ Patients included in the study are, however, encouraged to continue (adjusted) PR. The large number of biomarkers combined with the relatively small sample size and expected patient/AECOPD heterogeneity may challenge the identification of statistically significant biomarker associations. Yet, the extensive patient characterisation and explorative nature of this study allow for hypotheses to be prospectively tested in future studies. Finally, despite the intensive sampling and patient monitoring intended to capture exacerbations, other unknown predictors not assessed in the study may be missed.

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Contributors KWS: contributed substantially to the conception, design and acquisition of the study, drafted the work, approved the final version to be published and agrees to be accountable for all aspects of the work. AD, JB, KO, UG, BRS, TK, MAS, SHW, FMEF: contributed substantially to the conception, design and acquisition of the study, critically revised the work for important intellectual content, approved the final version to be published and agrees to be accountable for all aspects of the work. SMJVK: contributed substantially to the conception and design of the study, critically revised the work for important intellectual content, approved the final version to be published and agrees to be accountable for all aspects of the work. DP: contributed substantially to the acquisition of the study, approved the final version to be published and agrees to be accountable for all aspects of the work. SOS: contributed substantially to the design of the study, critically revised the work for important intellectual content, approved the final version to be published and agrees to be accountable for all aspects of the work. FMEF is the principal investigator of the MARKED study.

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Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

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Data availability statement The dataset is currently being generated and will be analysed upon completion of the final inclusion.

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