PROTOCOL

A 16-week, randomized, placebo-controlled, double blind, and parallel group trial to assess the anti-inflammatory effects of Roflumilast in chronic obstructive pulmonary disease

The ROBERT study

(Roflumilast Biopsy European Research Trial)

Sponsor: Takeda Development Centre Europe Ltd.

Study Number: RO-2455-402-RD

IND Number: Not Applicable **EudraCT Number:** 2011-000582-13

Compound: Roflumilast

Date: **Amendment Number:** 12

Amendment History:

Protocol Version	**Protocol Amendment Number**	**Date**	**Country**
Version: 1.0 (initial version)**			Global
	1*		Germany
	2*		Italy
	3*		Poland
	4*		Germany
	5*		Global
Version: 2.0**	Incl Am 5		Global
Version: 2.0**	Incl Am 1, 4, 5		Germany
Version: 2.0**	Incl Am 3, 5		Poland

	6*		Global
	7*		Germany
	8*		UK
	9*		Global
Version: 3.0**	Incl Am 5, 9		Global
Version: 3.0**	Incl Am 1, 4, 5, 9		Germany
Version: 3.0**	Incl Am 3, 5, 9		Poland
	10*		Global
Version 4.0**	Incl Am 5, 9, 10		Global
Version 4.0**	Incl Am 1, 4, 5, 9, 10		Germany
Version 4.0**	Incl Am 3, 5, 9, 10		Poland
	11*		Global
Version 5.0**	Incl all amendments		Global
Version 6.0**	Incl. all amendments		Global

1.0 ADMINISTRATIVE INFORMATION

1.1 Contacts

A separate contact information list will be provided to each site.

Investigators at Takeda sponsored sites will be provided with emergency medical contact information cards to be carried by each subject.

General advice on protocol procedures should be obtained through the monitor assigned to the study site. Information on service providers is given in Section 3.1 and relevant guidelines provided to the site.

Contact Type/Role	Global Contact
Serious adverse event and pregnancy reporting	Global Pharmacovigilance Takeda Development Centre Europe Ltd. Please send reports to: or
Medical Monitor (medical advice on protocol and compound)	Associate Medical Director, Pharmacovigilance PPD Bulgaria EOOD Hotline: SAE Fax:
Responsible Medical Officer (carries overall responsibility for the conduct of the study)	Medical Director, Clinical Sciences, CVM and Respiratory Takeda Development Centre Europe Limited

1.2 Approval

REPRESENTATIVES OF TAKEDA

This study will be conducted with the highest respect for the individual participants in accordance with the requirements of this clinical study protocol and also in accordance with the following:

- The ethical principles that have their origin in the Declaration of Helsinki.
- International Conference on Harmonisation E6 Good Clinical Practice: Consolidated Guideline.
- All applicable laws and regulations, including, without limitation, data privacy laws, clinical trial disclosure laws, and regulations.

SIGNATURES

The signature of the responsible Takeda medical officer (and other signatories, as applicable) can be found on the signature page.

Electronic Signatures may be found on the last page of this document.

Date	, Date
Clinical Sciences, Medical Director	Analytical Science, Principal Statistician,.
, Date	Date
Global Pharmacovigilance, Medical Director, Medical Safety CV / Metabolic TA	Clinical Operations, Clinical Study Manager

1.2 Approval

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Date	Date
Clinical Sciences, Medical Director	Analytical Science, Principal Statistician,.
Date	Date
Global Pharmacovigilance, Medical Director, Medical Safety CV / Metabolic TA	Clinical Operations, Clinical Study Manager

, Date
GCDT Lead, Executive Medical Director, CVM & Respiratory

_____

Date

GCDT Lead, Executive Medical Director, CVM & Respiratory

INVESTIGATOR AGREEMENT

I confirm that I have read and that I understand this protocol, the Investigator's Brochure, and any other product information provided by the sponsor. I agree to conduct this study in accordance with the requirements of this protocol and also to protect the rights, safety, privacy, and well-being of study subjects in accordance with the following:

- The ethical principles that have their origin in the Declaration of Helsinki.
- International Conference on Harmonisation, E6 Good Clinical Practice: Consolidated Guideline.
- All applicable laws and regulations, including, without limitation, data privacy laws and regulations.
- Regulatory requirements for reporting serious adverse events defined in Section 10.2 of this protocol.
- Terms outlined in the Clinical Study Site Agreement.
- Appendix B – Responsibilities of the Investigator

I further authorize that my personal information may be processed and transferred in accordance with the uses contemplated in Appendix D of this protocol.

Signature of Investigator Date

Investigator Name (print or type)

Investigator's Title

Location of Facility (City, State/Provence)

Location of Facility (Country)

1.3 Protocol Amendment 12 Summary of Changes

This document describes the changes in reference to the Protocol Incorporating Amendment No. 11.

The primary purpose of this amendment is to update the protocol to clarify requirements which were inadvertently omitted from the previous version of the protocol. The following is a brief summary of the changes made in the amendment:

1. Subjects should not take any food or drink (fast) for at least 8 hours prior to each study visit. Subjects may take their study medication with water on study visit days if this is considered acceptable in the clinical judgement of the investigator.
2. Smoking is prohibited for 4 hours before any study procedures.
3. Strenuous exercise is not permitted 8 hours prior to each study visit.
4. Clarify location of bronchial biopsy brushings at visit 2 and visit 6.
5. Minor typographical errors and updating of study team members, including Medical Responsible.

Full details on changes of text are given in Appendix L.

TABLE OF CONTENTS

1.0 ADMINISTRATIVE INFORMATION 3
1.1 Contacts....3
1.2 Approval 4
1.3 Protocol Amendment 11 Summary of Changes....7
2.0 STUDY SUMMARY....13
3.0 STUDY REFERENCE INFORMATION 16
3.1 Study-Related Responsibilities....16
3.2 Coordinating Investigator 16
3.3 List of Abbreviations 17
3.4 Corporate Identification....19
4.0 INTRODUCTION 20
4.1 Background....20
4.2 Trial Treatment: Roflumilast 20
4.3 Rationale for the Proposed Study 22
5.0 STUDY OBJECTIVES AND ENDPOINTS....23
5.1 Objectives 23
5.1.1 Primary Objective(s)....23
5.1.2 Secondary Objectives....23
5.1.3 Additional Objectives 23
5.2 Endpoints 23
5.2.1 Primary Endpoints 23
5.2.2 Secondary Endpoints 23
5.2.3 Additional Endpoints....25
6.0 STUDY DESIGN AND DESCRIPTION....26
6.1 Study Design....26
6.2 Justification for Study Design, Dose, and Endpoints 27
6.3 Premature Termination or Suspension of Study or Investigational Site....30
6.3.1 Criteria for Premature Termination or Suspension of the Study 30
6.3.2 Criteria for Premature Termination or Suspension of Investigational Sites....30
6.3.3 Procedures for Premature Termination or Suspension of the Study or the Participation of Investigational Site(s)....30
7.0 SELECTION AND DISCONTINUATION/WITHDRAWAL OF SUBJECTS....31
7.1 Inclusion Criteria 31
7.2 Exclusion Criteria 31

7.3 Randomization Criteria....34
7.4 Re-Enrolment....34
7.5 Diet, Fluid, Activity Control....34
7.6 Excluded Medications....35
7.7 Allowed Concomitant Medications....35
7.8 Criteria for Discontinuation or Withdrawal of a Subject....36
7.9 Procedures for Discontinuation or Withdrawal of a Subject....37
8.0 CLINICAL TRIAL MATERIAL MANAGEMENT....38
8.1 Study Medication and Materials....38
8.1.1 Dosage Form, Manufacturing, Packaging, and Labeling....38
8.1.2 Storage....39
8.1.3 Dose and Regimen....39
8.1.4 Overdose....39
8.2 Investigational drug Assignment and Dispensing Procedures....40
8.3 Investigational Drug Blind Maintenance....40
8.4 Unblinding Procedure....41
8.5 Accountability and Destruction of Sponsor-Supplied Drugs....41
9.0 STUDY PLAN....43
9.1 Study Procedures....43
9.1.1 Informed Consent Procedure....43
9.1.2 Demographics, Medical History, and Medication History Procedure....43
9.1.3 Physical Examination Procedure....43
9.1.4 Weight, Height....44
9.1.5 Vital Sign Procedure....44
9.1.6 Primary Efficacy Measurement....44
9.1.7 Documentation of Concomitant Medications....44
9.1.8 Documentation of Concurrent Medical Conditions....45
9.1.9 Procedures for Clinical Laboratory Samples....45
9.1.10 Contraception and Pregnancy Avoidance Procedure....47
9.1.11 Pregnancy....48
9.1.12 ECG Procedure....48
9.1.13 Documentation of Screen Failure....49
9.1.14 Documentation of Randomization....49
9.1.15 Bronchoscopy....49
9.1.16 Spirometry/Pulmonary Function Test....51

9.1.17 Sputum Induction....52
9.1.18 Inflammatory Biomarkers in Blood Serum....54
9.2 Monitoring Subject Treatment Compliance....54
9.3 Schedule of Observations and Procedures....55
9.3.1 Screening / Visit 0 (V0)....56
9.3.2 Run-in / Visit V1 (4 weeks after baseline visit V0)....57
9.3.3 Randomization / Visit V2 (6 weeks after baseline visit V0)....57
9.3.4 Treatment Phase....58
9.3.5 Final Visit or Early Termination....60
9.3.6 Follow-up / Visit V7 (≤2 weeks after visit V6)....61
9.3.7 Post Study Care....61
9.4 Biological Sample Retention and Destruction....61
10.0 PRETREATMENT EVENTS AND ADVERSE EVENTS....63
10.1 Definitions....63
10.1.1 AEs....63
10.1.2 Additional Points to Consider for AEs....63
10.1.3 SAEs....65
10.1.4 Severity of AEs....66
10.1.5 Causality of AEs....66
10.1.6 Relationship to Study Procedures....66
10.1.7 Start Date....67
10.1.8 Stop Date....67
10.1.9 Action Concerning Study Medication....67
10.1.10 Outcome....67
10.2 Procedures....68
10.2.1 Collection and Reporting of AEs....68
10.2.2 Collection and Reporting of SAEs....69
10.3 Follow-up of SAEs....69
10.3.1 Safety Reporting to Investigators, IRBs or IECs, and Regulatory Authorities....69
11.0 STUDY-SPECIFIC COMMITTEES....71
11.1 Steering Committee....71
12.0 DATA HANDLING AND RECORDKEEPING....72
12.1 CRFs (Electronic and Paper)....72
12.2 Record Retention....72
13.0 STATISTICAL METHODS....74

13.1 Statistical and Analytical Plans....74
13.1.1 Analysis Sets....74
13.1.2 Analysis of Demographics and Other Baseline Characteristics....75
13.1.3 Efficacy Analysis....75
13.1.4 Other Analyses....78
13.1.5 Safety Analysis....78
13.2 Interim Analysis and Criteria for Early Termination....79
13.3 Determination of Sample Size....80
14.0 QUALITY CONTROL AND QUALITY ASSURANCE....82
14.1 Study-Site Monitoring Visits....82
14.2 Protocol Deviations....82
14.3 Quality Assurance Audits and Regulatory Agency Inspections....82
15.0 ETHICAL ASPECTS OF THE STUDY....83
15.1 IRB and/or IEC Approval....83
15.2 Subject Information, Informed Consent, and Subject Authorization....84
15.3 Subject Confidentiality....85
15.4 Publication, Disclosure, and Clinical Trial Registration Policy....85
15.4.1 Publication and Disclosure....85
15.4.2 Clinical Trial Registration....86
15.4.3 Clinical Trial Results Disclosure....86
15.5 Insurance and Compensation for Injury....86
16.0 REFERENCES....87

LIST OF IN-TEXT TABLES

Table 8.a Dispensing of Trial Treatment....39
Table 9.a Clinical Laboratory Tests....45
Table 10.a Takeda Medically Significant AE List....66

LIST OF IN-TEXT FIGURES

Figure 6.a Schematic of Study Design....27

LIST OF APPENDICES

Appendix A Schedule of Study Procedures 90
Appendix B Responsibilities of the Investigator.....90
Appendix C Elements of the Subject Informed Consent 94
Appendix D Investigator Consent to Use of Personal Information.....97
Appendix E Laboratory and Vital Sign Alert Values (Sponsor defined).....98
Appendix F Bronchoscopy Manual 99
Appendix G Biopsy Laboratory Manual 102
Appendix H Sputum Laboratory Manual 120
Appendix I Microbiome Sub study: The interaction between changes in the lung microbiota, airway inflammation and mucin production resulting from roflumilast therapy.130
Appendix J For Germany: The TRANSCRIPTOM Sub study only valid for the site of Professor Klaus Rabe.....143
Appendix K For UK: The Airway Reflux Sub study only valid for the site of Professor Alyn H Morice 146
Appendix L Detailed Description of Amendments to Text 153

2.0 STUDY SUMMARY

<table>
<tr><td colspan="2">Name of Sponsor(s):
Takeda Development Centre Europe Ltd.</td><td colspan="2">Compound:
Roflumilast</td></tr>
<tr><td>Title of Protocol: A 16-week, randomized, placebo-controlled, double blind, and parallel group trial to assess the anti-inflammatory effects of Roflumilast in chronic obstructive pulmonary disease
The ROBERT study</td><td>IND No.:
Not Applicable</td><td colspan="2">EudraCT No.:
2011-000582-13</td></tr>
<tr><td colspan="2">Study Number: RO-2455-402-RD</td><td colspan="2">Phase: III Clinical Exploratory</td></tr>
<tr><td colspan="4">Study Design:
This is a phase III, randomized, placebo-controlled, double blind, and parallel group trial to assess the anti-inflammatory effects of Roflumilast in chronic obstructive pulmonary disease. The duration is 24 weeks maximum, and consists of the following periods:
• Single-blind placebo run-in period (6 weeks) with visits scheduled at weeks: -6 (V0), -2 (V1) and 0 (V2, randomization visit).
• Double-blind treatment period (16 weeks) with visits scheduled at 6, 14, and 16 weeks of treatment (V4-V6). An additional visit V3 will have to be performed within two weeks after bronchoscopy/bronchial biopsy purely as a safety visit. The exact timing of this safety visit will be determined by the Investigator.
• Safety follow-up. All adverse events (AEs) will be followed up to 30 days after the double-blind treatment period has been completed. An additional safety visit V7 will be scheduled within 2 weeks after the second bronchoscopy. The exact timing of the safety visit will be determined by the Investigator.</td></tr>
<tr><td colspan="4">Primary Objectives:
• To investigate the effect of Roflumilast 500 µg tablets once daily versus placebo on inflammatory cells (CD8+) in bronchial biopsy tissue specimens.</td></tr>
<tr><td colspan="4">Secondary Objectives:
• To investigate inflammatory cells and parameters in bronchial biopsy tissue, sputum and blood serum.
• Safety status will be assessed by vital signs, physical examination (including electrocardiogram [ECG]), clinical laboratory tests, and monitoring of adverse events.</td></tr>
<tr><td colspan="4">Subject Population: Patients with COPD (GOLD stage II-III, in Germany stage II only)</td></tr>
<tr><td colspan="2">Number of Subjects:
A total of 150 Subjects is to be randomized (1:1) into two treatment arms (Roflumilast 500 µg or placebo).</td><td colspan="2">Number of Sites:
Estimated total: Approximately 25 sites in Europe.</td></tr>
<tr><td colspan="2">Dose Level(s):
• Single-blind baseline period (V0-V2): one placebo tablet once daily.
• Double-blind treatment period (V2 [randomization]-VE): one tablet containing placebo or Roflumilast 500 µg once daily.</td><td colspan="2">Route of Administration:
Roflumilast: oral
Placebo: oral</td></tr>
</table>

Duration of Treatment:	**Period of Evaluation:**
Single-blind placebo run-in period: 6 weeks Double-blind treatment period: 16 weeks	24 weeks (max.)

Main Criteria for Inclusion:

Current or former smokers with history of COPD (according to GOLD 2009) for at least 12 months prior to baseline visit V0 associated with chronic productive cough for at least three months in each of the two years prior to baseline visit V0 (with other causes of productive cough excluded), age 40-80 years old, Post-bronchodilator 30% ≤FEV1 ≤80% predicted (GERMANY ONLY: Post-bronchodilator 50% ≤FEV1 ≤80% predicted) and Post-bronchodilator FEV1/FVC ratio ≤70%.

Main Criteria for Exclusion:

Subjects who are clinically instable, or have unresolved respiratory infection, or known alpha-1-antitrypsin deficiency or other relevant lung disease, or other situations which should be excluded based on ethical considerations.

Main Criteria for Evaluation and Analyses:

The primary endpoint for this study is the number of inflammatory cells CD8+ in bronchial biopsy tissue specimen (sub-mucosa) measured at randomization visit V2 and at the end of the intervention period (V6).

The secondary endpoints for this study are:

- Cell count in biopsied material (sub-mucosa): CD68+ / Neutrophils / CD4+ / CD45+
- Cell count in biopsied material (bronchial epithelium): CD8+ / CD68+
- Total and differential cell counts in induced sputum: Neutrophils / Macrophages / Eosinophils / Lymphocytes
- Concentration of inflammatory biomarkers in sputum
- Concentration of inflammatory biomarkers and different metabolites in blood serum
- Pulmonary Function Changes

Statistical Considerations:

Analyses will be based on the following sets: total set, safety set, full analysis set, valid cases set.

The **primary variable** relates to pulmonary inflammation expressed as inflammatory CD8+ cell counts per mm^2 in sub-mucosal bronchial biopsy tissue specimen measured before and after the double-blind treatment period (i.e. at V2 and at V6). CD68+ cell counts per mm^2 in sub-mucosal bronchial biopsy tissue specimen at V2 and at V6 is the **key secondary variable**.

Roflumilast/placebo comparisons with respect to CD8+ and CD68+ will be done via a multiple test procedure such that the family-wise error rate of 5% is controlled in the strong sense. The two null hypotheses are: equal CD8+ counts/mm^2 and equal CD68+ counts/mm^2 on Roflumilast and placebo. These null hypotheses will be ordered, so that the CD8+ comparison comes first and the CD68+ comparison comes second. If the comparison with respect to CD8+ is significant at the nominal level alpha = 5%, the corresponding null hypothesis will be rejected and the CD68+ comparison will be performed in a confirmatory way (otherwise confirmatory testing stops). This will again be done at nominal level alpha = 5%. If significant (after a significant result in the first comparison), the corresponding null hypothesis will be rejected. If the first comparison is not significant at the nominal 5% level, then no null hypothesis must be rejected.

The component tests of the multiple test on CD8+ and CD68+ will be based on Poisson regression models with CD8+ (CD68+) at visit V6 as dependent variable and treatment and baseline value of the respective dependent variable as covariates. A dispersion parameter and an offset (= bronchoscopy sampling area) will be taken into account.

Sample Size Justification:

Extensive literature search/review and experimental data provided by the Steering Committee (SC) members reveals significant differences exist between reports published by various groups, which may be attributable to different patient populations, bronchoscopy/tissue harvesting/processing methods and statistical methods employed.

Based on SC members' opinion, a universally recognized level of clinical relevance regarding the first primary variable (sub-mucosa CD8+ cells) has not yet been agreed upon within the scientific community of chest physicians and pathologists. But it has been indicated by SC members that a 30% improvement on Roflumilast over placebo may be of clinical relevance.

For the primary variable (subepithelial CD8+ cell count/mm^2) sample size is calculated under a Poisson regression model with an assumed dispersion parameter. Sample size was calculated according to formula (4.3) of Yee, 1998 [28]. Further assumptions were the following: 1:1 randomization, two-sided alpha = 0.05, power = 0.90, 0.85, 0.80, event rate on Roflumilast = 200 cells/mm^2, event rate on placebo 285 cells/mm^2. Such a reduction of event rates would correspond to an improvement of ca. 30% on Roflumilast (placebo event rate corresponds to 100%). Given the uncertainty in the assumptions around the mean area examined and the counts per mm^2, Takeda may conduct a blinded pooled assessment of these quantities and revise the sample size to ensure the study remains adequately powered.

Historical research also indicates that drop-out rates (expressed as a percentage of enrolled Subjects) may be as high as 30%. To have a conservative estimate a 30% drop-out rate is assumed in this trial. Thus, the trial may end up with 105 Subjects having two bronchoscopies.

Following the analysis of literature data, SC members' opinion and for feasibility reasons the total number of enrolled Subjects has been capped at 150 in total. Thus, with a dispersion of 25 and a tissue area of at least 0.3 mm^2 and further assumptions as above, the trial would have a high power (ca. 90%) to detect treatment differences.

3.0 STUDY REFERENCE INFORMATION

3.1 Study-Related Responsibilities

The sponsor will perform all study-related activities with the exception of those identified in the Clinical Study Supplier List. The identified vendors in the template for specific study-related activities will perform these activities in full or in partnership with the sponsor.

3.2 Coordinating Investigator

Takeda will select a Signatory Coordinating Investigator from the investigators who participate in the study. Selection criteria for this investigator will include significant knowledge of the study protocol, the study medication, their expertise in the therapeutic area and the conduct of clinical research as well as study participation. The Signatory Coordinating Investigator will be required to review and sign the clinical study report and by doing so agrees that it accurately describes the results of the study.

Pneumologisches Forschungsinstitut an der LungenClinic Grosshansdorf GmbH

3.3 List of Abbreviations

AE	Adverse Event
ALT	Alanine Aminotransferase
am	ante meridiem
AP	Alkaline Phosphatase
APTT	Activated Partial Thromboplastin Time
ANCOVA	Analysis of Covariance
AR	Adverse Reaction
AST	Aspartate Aminotransferase
ATC	Anatomical Therapeutic Chemical
ATS	American Thoracic Society
BDRM	Blinded Data Review Meeting
BMI	Body Mass Index
BP	Blood Pressure
CA	Competent Authority
CABG	Coronary Artery Bypass Surgery
cAMP	Cyclic Adenosine Monophosphate
CCSI	Company Core Safety Information
CD	Cluster of Differentiation
CK-MB	Creatine Kinase-MB (Muscle, Brain)
CHMP	Committee for Medicinal Products for Human Use
COPD	Chronic Obstructive Pulmonary Disease
CPMP	Committee for Proprietary Medicinal Products
CRA	Clinical Research Assistant
CRF	Case Report Form
CRO	Contract Research Organization
CT	X-ray Computed Tomography
ECG	Electrocardiogram
EM(E)A	European Medicines Agency
ERS	European Respiratory Society
EU	European Union
EWP	Efficacy Working Party
FAS	Full Analysis Set
FEV_1	Forced Expiratory Volume in the First Second
FVC	Forced Vital Capacity (Expiratory)
FU	Follow-Up Visit
gamma-GT	gamma-Glutamyl-Transpeptidase
GCP	Good Clinical Practice
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
GMP	Good Manufacturing Practice

GOLD	Global Initiative for Chronic Obstructive Lung Disease
HIV	Human Immunodeficiency Virus
HR	Heart Rate
ICH	International Conference on Harmonisation
ICS	Inhaled Corticoid Steroid
IDS	International Drug Safety
IEC	Independent Ethics Committee
IL	Interleukin
INR	International Normalized Ratio
IRB	Institutional Review Board
IND	Investigational New Drug Application
ITT	Intention to Treat
IVRS	Interactive Voice Response System
IWRS	Interactive Web Response System
LABA	Long-Acting β_2-Agonist (Long-acting beta-2-adrenoceptor agonists)
LAMA	Long-Acting Muscarinic Antagonist
LOCF	Last Observation Carried Forward
LMWH	Low Molecular Weight Heparin
MAP	Multi-Analyte Profile, Multi-Analyte Profiling
MCP	Monocyte Chemotactic Protein
MDI	Metered-Dose Inhaler
MedDRA	Medical Dictionary for Regulatory Activities
MH	Medical History
mmHg	Millimetre of Mercury
MMP	Matrix Metalloproteinase
NCR	"No Carbon Required" Paper (Carbonless Copy Paper)
NSAID	Non-Steroidal Anti-Inflammatory Drug
NYHA	New York Heart Association Functional Classification
o.d.	once daily
PDE	Phosphodiesterase
PP	Per Protocol
Prn	Pro re nata (Lat.; “as needed” or “as the situation arises”)
PT	MedDRA Preferred Term
PT	Prothrombin Time
PTCA	Percutaneous Transluminal Coronary Angioplasty
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RBM	Rules-Based Medicine, Inc.
SABA	Short-Acting β_2-Agonist
SAE	Serious Adverse Event
SAF	Safety Set

SAMA	Short-Acting Muscarinic Antagonist
SAP	Statistical Analysis Plan
SC	Steering Committee
SDV	Source Data Verification
SOC	System Organ Class
SOP	Standard Operating Procedure
SUSAR	Suspected Unexpected Serious Adverse Reaction
TIMP	Tissue Inhibitor of Metalloproteinase
TNF	Tumour Necrosis Factor
TT	Thromboplastin Time
ULNR	Upper Limit of Normal Range
V	Visit
VCS	Valid Cases Set
VE	Visit End (V6)
VEGF	Vascular Endothelial Growth Factor
WBC	White Blood Cell (Leukocyte)
WHO	World Health Organization
WHODD	WHO Drug Dictionary
WPDs	Working Practice Documents

3.4 Corporate Identification

TDC Japan	Takeda Development Center Japan
TDC Asia	Takeda Development Center Asia, Pte Ltd.
TDC Europe	Takeda Development Centre Europe Ltd.
TDC Americas	Takeda Development Center Americas, Inc.
TDC	TDC Japan, TDC Asia, TDC Europe and/or TDC Americas, as applicable
Takeda	TDC Japan, TDC Asia, TDC Europe and/or TDC Americas, as applicable

4.0 INTRODUCTION

4.1 Background

Chronic obstructive pulmonary disease (COPD) is a progressive disease characterised primarily by a pulmonary component but is also associated with significant extrapulmonary (systemic) consequences. Its pulmonary component is characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases, primarily caused by cigarette smoking [1, 2, 3].

A specific pattern of inflammation in the airway and lung parenchyma of COPD patients could be observed, with increased number of monocyte/macrophages (CD68+), T-lymphocytes with the predominance of cytotoxic CD8+T-cells and in more severe stage of the disease B-lymphocytes with an increased number of neutrophils in the lumen [3, 4]. The concentration of several inflammatory mediators such as interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α), neutrophil elastase and E-selectin is increased. Inflammatory mediators are being produced not only by the inflammatory, but the structural cells of the airways and the lung as well [5].

The chronic airflow limitation is caused by a combination of small airway disease (obstructive bronchiolitis) and parenchymal destruction (emphysema), the relative contributions of which vary from patient to patient. Despite recent progress in understanding the pathophysiology and treatment of the disease, COPD still presents a major health problem as an important cause of morbidity, mortality, and burden on health care systems worldwide [2]. Symptomatic COPD patients at risk of exacerbations present the most important unmet medical need [2]. Patients with frequent exacerbations have not only a reduced quality of life but also a more rapid decline in lung function [6].

4.2 Trial Treatment: Roflumilast

Roflumilast is a phosphodiesterase 4 (PDE4) inhibitor, a non-steroid, anti-inflammatory agent designed to target both the systemic and pulmonary inflammation associated with COPD. The mechanism of action is the inhibition of PDE4, the major cyclic adenosine monophosphate (cAMP)-metabolising enzyme in structural and inflammatory cells important in the pathogenesis of COPD. Inhibition of PDE4 increases intracellular cAMP and typically leads to an anti-inflammatory effect. In line, Roflumilast and its major active metabolite, Roflumilast N-oxide, demonstrated potent anti-inflammatory effects in animal as well as clinical studies.

The efficacy and safety of Roflumilast in COPD have been investigated in a variety of clinical phase II and III studies in patients with moderate to very severe COPD. In all studies Roflumilast consistently increased lung function versus placebo.

In the two pivotal randomized, multi-national, 1-year trials (BY217/M2-124 and BY217/M2-125), Roflumilast 500 µg once daily was shown to be effective in the treatment of COPD associated with chronic bronchitis in patients at risk of frequent exacerbations [7]. Long-Acting ß$_2$-Agonists

(LABAs) were allowed in these studies and were used in approximately 50% of the trial population. In these studies Roflumilast significantly improved lung function compared to placebo (pre-bronchodilator Forced Expiratory Volume in the first second [FEV_1], between-treatment difference 39 mL and 58 mL, primary endpoint). In addition, moderate or severe exacerbations (co-primary endpoint) were significantly reduced with Roflumilast compared with placebo by 15% (BY217/M2-124) and 19% (BY217/M2-125) after 1 year. These effects were similar, independent of previous treatment with Inhaled Corticosteriods (ICS) or underlying treatment with LABAs.

The results of the two pivotal studies were confirmed and extended by two six-months studies (trials BY217/M2-127 and BY217/M2-128) evaluating the effect of Roflumilast as add-on treatment to long-acting bronchodilators [8]. In these studies, pre-bronchodilator FEV_1 (primary endpoint in both studies) was significantly improved by 49 mL beyond the bronchodilator effect of concomitant treatment with salmeterol in trial BY217/M2-127 and by 80 mL incremental to concomitant treatment with tiotropium in trial BY217/M2-128. Although not designed to show treatment effects on exacerbations, both studies suggested beneficial effects of Roflumilast in this respect.

Further two 1-year trials (M2-111 and M2-112) similar to the pivotal studies but using a different patient population also showed significant effects of Roflumilast in terms of lung function and indicated beneficial effects in terms of exacerbations. These studies also indicated that the effects of Roflumilast are independent of ICS (which was prohibited in the pivotal studies).

In all studies performed, Roflumilast was generally safe and well tolerated under long-term exposure (once daily administration for up to one year). Diarrhoea, weight decreased, nausea, abdominal pain and headache, were the most frequently reported AEs and considered associated with intake of Roflumilast. With respect to the event of weight decrease, weight measurements in the two pivotal studies showed a larger mean weight loss in Roflumilast-treated patients compared to placebo-treated patients which was however reversible upon Roflumilast withdrawal [9]. Roflumilast was also associated with an increased risk of psychiatric disorders such as insomnia, anxiety, nervousness and depression. Rare instances of suicidal ideation and behaviour, including completed suicide, have been observed in clinical trials. A causal relationship to Roflumilast could neither be established nor excluded due to the low number of cases and the known increased risk of COPD patients for depression and suicides.

Roflumilast (DAXAS®) was approved in the European Union by the European Commission on 5 July 2010 for the maintenance treatment of severe COPD (FEV_1 post-bronchodilator less than 50% predicted) associated with chronic bronchitis in adult patients with a history of frequent exacerbations as add on to bronchodilator treatment.

In the U.S.A., Roflumilast (DALIRESP®) was approved by FDA on 28 February 2011 as a treatment to reduce the risk of COPD exacerbations in patients with severe COPD associated with chronic bronchitis and a history of exacerbations.

Further details on Roflumilast are available in the Investigator's brochure [9].

4.3 Rationale for the Proposed Study

Clinical data have shown that Roflumilast improves pulmonary function and decreases the exacerbation rates of patients suffering from COPD. In numerous in-vitro and in-vivo pre-clinical models the mode of action of Roflumilast has been shown to be anti-inflammatory. However, clinical data of effects in the lungs as target organ as well as systemic effects are limited. It is hypothesized that Roflumilast would reduce the characteristic airway inflammation in patients with COPD (GOLD stage II-III, **in Germany stage II only**) treated for 16 weeks in a double blind, parallel group, randomized, placebo controlled trial. Inflammatory cell counts and several inflammatory markers in endobronchial biopsy specimens, induced sputum, and blood serum will be examined prior to and subsequent to Roflumilast treatment.

The trial will not be statistically powered to investigate any clinical outcome measures with regard to the effectiveness of COPD treatment. However, the results of the pulmonary function tests will be used to better characterize patient population.

5.0 STUDY OBJECTIVES AND ENDPOINTS

5.1 Objectives

5.1.1 Primary Objective(s)

- To investigate the effect of Roflumilast 500 µg tablets once daily versus placebo on inflammation parameters in bronchial biopsy tissue specimen.

5.1.2 Secondary Objectives

- To investigate inflammation parameters in sputum and blood serum.
- Safety status will be assessed by vital signs, physical examination (including electrocardiogram [ECG]), clinical laboratory tests, and monitoring of adverse events.

5.1.3 Additional Objectives

To assess the substudies as specified in Appendices I, J and K.

- The interaction between changes in the lung microbiota, airway inflammation and mucin production resulting from roflumilast therapy (Appendix I)
- Gene expression profiling of inflammatory sputum cells in COPD – an exploratory research approach to study the molecular anti-inflammatory treatment effects of Roflumilast (Appendix J).
- An exploratory research approach to study the changes of the degree of airway reflux (Appendix K).

5.2 Endpoints

5.2.1 Primary Endpoints

The primary endpoint will be the number of CD8+ inflammatory cells in bronchial biopsy tissue specimen (sub-mucosa) measured at randomization visit V2 and at the end of the intervention period (V6).

5.2.2 Secondary Endpoints

Secondary endpoints will be the following parameters measured at visit V1 or V2 (depending on parameter) and at the end of the intervention period (V6):

Biopsy Material

- Cell count in biopsied material (sub-mucosa) [cells/mm^2]:
  1) CD68+
  2) Neutrophils

3) CD4+
4) CD45+

- Cell count in biopsied material (bronchial epithelium) [cells/mm^2]:
  1) CD8+
  2) CD68+

Induced Sputum

- Total and differential cell counts in induced sputum (absolute [cells/mL] and percentage [%]):
  1) Neutrophils
  2) Macrophages
  3) Eosinophils
  4) Lymphocytes
- Concentration of inflammatory biomarkers in sputum:
  1) Inflammatory mediators (Human InflammationMAP® v.1; Rules-Based Medicine, Inc. (RBM))[1]

Blood Serum

- Concentration of inflammatory biomarkers in blood serum: Inflammatory mediators (Human InflammationMAP® v.1; Rules-Based Medicine, Inc. (RBM))[1] Measurement of a panel of different metabolites in blood serum
- Measurement of a panel of different metabolites in blood serum

Pulmonary Function Changes in the Course of the Trial

- Change from randomization (V2) over 16 weeks of treatment for:
  1) Forced expiratory volume in the first 1 second (FEV_1 [L])
  2) Forced vital capacity (FVC [L])
  3) FEV_1/FVC [%]

[1] Includes: Alpha-1 Antitrypsin, Alpha-2 Macroglobulin, Beta-2 Microglobulin, Brain-Derived Neurotrophic Factor, C Reactive Protein, Complement 3, Eotaxin, Factor VII, Ferritin, Fibrinogen, GM-CSF, Haptoglobin, Intercellular Adhesion Molecule-1, Interferon gamma, Interleukin-1 alpha, Interleukin-1 beta, Interleukin-1 receptor antagonist, Interleukin-2, Interleukin-3, Interleukin-4, Interleukin-5, Interleukin-6, Interleukin-7, Interleukin-8, Interleukin-10, Interleukin-12 p40, Interleukin-12 p70, Interleukin-15, Interleukin-17, Interleukin-23, Matrix metalloproteinase type 2, Matrix metalloproteinase type 3, Matrix metalloproteinase type 9, Macrophage Inhibitory Protein-1 alpha, Macrophage Inhibitory Protein-1 beta, Monocyte Chemotactic Protein-1, RANTES, Stem Cell Factor, Tissue Inhibitor of Metalloproteinase, Tumor Necrosis Factor alpha, Tumor Necrosis Factor beta, Tumor Necrosis Factor receptor alpha 2, Vascular Cellular Adhesion Molecule type 1, Vascular Endothelial Growth Factor, von Willebrand's Factor, Vitamin D Binding Protein

5.2.3 Additional Endpoints

Safety

1) Adverse events
2) Changes in laboratory values
3) Changes in vital signs including blood pressure (BP) and heart rate (HR)
4) Changes in physical examination findings
5) Changes in body weight and body mass index (BMI)

6.0 STUDY DESIGN AND DESCRIPTION

6.1 Study Design

The total duration of this randomized, multicentre, phase III trial is 24 weeks maximum. It includes six weeks of single-blind placebo administration during the run-in period and 16 weeks of double-blind treatment, followed by a single safety visit (within two weeks) for subjects undergoing the second (final) bronchial biopsy.

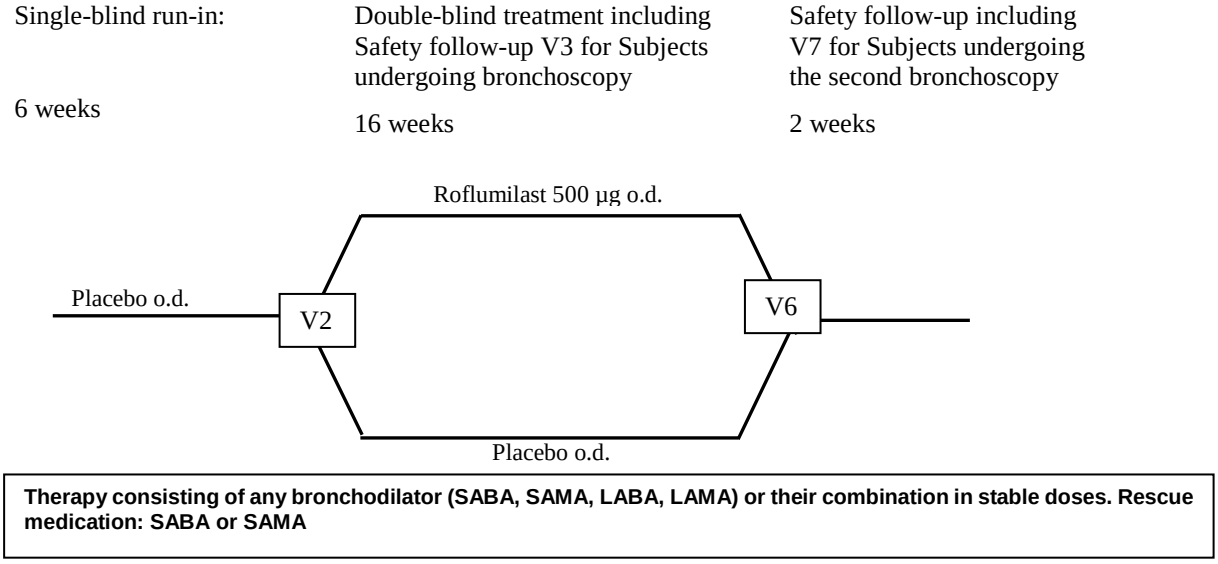
This trial will be performed in Chronic Obstructive Pulmonary Disease (COPD) patients (GOLD stage II-III, **in Germany stage II only**) and will include two parallel treatment arms (placebo and Roflumilast 500 µg once daily). A 1:1 randomization scheme will be used, i.e. Subjects will be allocated to Roflumilast 500 µg or placebo in equal proportions. Randomization will be stratified by concomitant LABA use.

The trial consists of the following periods:

- **Single-blind placebo run-in period** (6 weeks) with visits scheduled at weeks: -6 (V0), -2 (V1) and 0 (V2, randomization visit).
- **Double-blind treatment period** (16 weeks) with visits scheduled at 6, 14, and 16 weeks of treatment (V4-V6). An additional visit V3 will have to be performed within two weeks after bronchoscopy/bronchial biopsy purely as a safety visit. The exact timing of this safety visit will be determined by the Investigator.
- **Safety follow-up**. All adverse events (AEs) will be followed up to 30 days after the double-blind treatment period has been completed. An additional safety visit V7 will be scheduled within 2 weeks after the second bronchoscopy. The exact timing of the safety visit will be determined by the Investigator.

A schematic of the study design is included as Figure 6.a. A schedule of assessments is listed in Appendix A.

Figure 6.a Schematic of Study Design



Abbreviations: V2 – randomization visit and first bronchoscopy, V6 – visit at the end of double blind treatment period and second bronchoscopy, LABA – Long-Acting ß$_2$-Agonist, LAMA – Long-Acting Muscarinic Antagonist, SABA – Short-Acting ß$_2$-Agonist, SAMA – Short-Acting Muscarinic Antagonist, o.d. – once daily

6.2 Justification for Study Design, Dose, and Endpoints

It is hypothesized that Roflumilast would reduce the characteristic airway inflammation in patients with COPD (GOLD stage II-III, **in Germany stage II only**) treated for 16 weeks in a double blind, parallel group, randomized, placebo controlled trial.

Chronic obstructive pulmonary disease (COPD) is an inflammatory condition clinically characterized by progressive, not fully reversible airflow limitation and an accelerated decline in lung function [10]. The role of inflammatory cells in development of COPD was described and it could be shown that reductions of inflammatory cells correlated with clinical improvements [4, 11]. A specific pattern of inflammation in the airway and lung parenchyma of COPD patients could be observed, with increased number of monocyte/macrophages (CD68+), T-lymphocytes with the predominance of cytotoxic CD8+ T-cells and in more severe stage of the disease B-lymphocytes with an increased number of neutrophils in the lumen [4]. The concentration of several inflammatory mediators such as interleukin-8 (IL-8), tumour necrosis factor-α (TNF-α), neutrophil elastase and E-selectin is increased. Inflammatory mediators are being produced not only by the inflammatory, but the structural cells of the airways and the lung as well [5].

GOLD identifies a pressing need to develop agents that suppress the inflammation associated with COPD and prevent the progressive decline in lung function [2].

Roflumilast has shown in clinical studies to improve lung function and reduce COPD exacerbations. The purpose of the present trial is to better understand the anti-inflammatory effects of the drug Roflumilast in the target organ, the lungs, as well as in sputum and in the systemic circulation. Hence, this trial offers the opportunity to address these critical issues by ascertaining potentially important biomarkers and other surrogate endpoints to a clinical patient benefit, while simultaneously identifying anti-inflammatory treatment effects within patients suffering from COPD. Thereby better understanding of the pathophysiology of COPD, which will also be relevant for the development of further new anti-inflammatory COPD treatments. As primary and key secondary parameter the above described CD8+ T-cells and CD68+ cells were selected to be measured in the submucosa as most promising and relevant targets.

Bronchoscopy is a relatively safe procedure. Serious risks from bronchoscopy, such as an air leak or serious bleeding are reported in less than 5 % of procedures. Other risks associated with the procedure are as follows: bleeding, discomfort and coughing, reduced oxygen, lung leak or collapse (pneumothorax) [12]. Bronchoscopy has an invaluable place in airway research. Given that the safety of the volunteer Subject is of paramount importance, the conduct of the clinical trials should be based on well established methods [13]. The individual risk to the Subjects from the bronchoscopy/bronchial biopsy procedure is as minimized based on several principles implemented within the protocol. The bronchoscopy and bronchial biopsy will only be conducted at experienced investigational sites which have shown in previous clinical trials to be capable of performing bronchoscopies in a standardized and safe manner. The same predefined, standardized procedures will be followed, the sites will be individually trained on the trial related procedures, and the quality of the biopsied material will be validated for each site. Dedicated safety follow-up visits have been implemented in the trial schedule subsequent to each visit with a bronchoscopy assessment. In addition, the sputum collection sampling has been separated from the bronchoscopy visits in order to not perform too many interventions on the same day.

A treatment regimen with a once daily dose of Roflumilast 500 µg was effective and safe in several clinical trials assessing COPD patients of GOLD stages II-IV. DAXAS® (Roflumilast 500 µg) was approved in the European Union by the European Commission on 5 July 2010 for the maintenance treatment of severe COPD (FEV_1 post-bronchodilator less than 50% predicted) associated with chronic bronchitis in adult patients with a history of frequent exacerbations as add on to bronchodilator treatment. In the U.S.A., Roflumilast 500 µg (DALIRESP®) was approved by FDA on 28 February 2011 as a treatment to reduce the risk of COPD exacerbations in Subjects with severe COPD associated with chronic bronchitis and a history of exacerbations.

This is a randomized, double-blind, placebo-controlled trial. Since all Subjects will continue to receive their bronchodilator standard therapy (e.g. LABA and/or LAMA in stable doses) placebo-treatment does not bear any additional risk. The placebo arm is thus ethically justified. All Subjects will be encouraged to contact their Investigator in case of a deterioration of their disease.

After drop-out and/or end of trial treatment Subjects will be treated according to their individual needs and no longer under standardized conditions.

In this clinical trial spirometry assessments will be performed to characterize the disease stage and the disease progress of the enrolled COPD patients. Patients with moderate to severe stage of COPD (stage II and III) will be enrolled; however, no patients with COPD stage IV will be enrolled. (***GERMANY ONLY:*** Patients with moderate stage of COPD (stage II) will be enrolled; however, no patients with COPD stage III and IV.) It can be expected that COPD patients with GOLD stage II-III **(in Germany stage II only)** are in a stable health condition to safely undergo the bronchoscopy with bronchial biopsy and sputum collection during the trial. For the same reason patients with a history of a recent exacerbation will be excluded from trial participation.

For biomarker studies in clinical trials, it is important to have relatively easy access to the samples containing the biomarker. The collection of blood serum for analysis is based upon the hypothesis that low-grade systemic effects, including inflammation and oxidative stress, play an important role in the pathogenesis of COPD. A number of markers for systemic inflammation have been shown to be associated with smoking and reduced FEV_1 [14]. Just like blood, induced sputum offers an opportunity to obtain samples containing important disease characteristics with relatively easy access [15]. Many COPD patients produce sputum spontaneously, but because spontaneously produced sputum often contains a large proportion of dead cells, which complicates measurements, induced sputum is the present procedure of choice. The biomarkers provided in the Multi-Analyte Profile (MAP) Human InflammationMAP® v.1 (see Section 9.1.17.2) and their distribution will be analysed in blood serum and induced sputum. The cell counts for neutrophils, macrophages, eosinophils and lymphocytes in sputum will be analysed and will serve as a surrogate for airway inflammation. In a 4-week trial, Roflumilast treatment reduced the number of neutrophils and eosinophils, as well as soluble markers of neutrophilic and eosinophilic inflammatory activity in induced sputum samples of patients with COPD [16]. Additionally, it was recently shown that Roflumilast significantly reduced the endotoxin-evoked influx of neutrophils, eosinophils, and total cells into the bronchoalveolar compartment in healthy volunteers [17]. In the present trial, for the first time treatment effects of Roflumilast can be associated to biomarkers in blood and sputum, and effects in the target organ, the lungs.

This trial includes a six-week single-blind run-in period to assess Subjects' compliance and for reasons of standardization. The treatment phase is for 16 weeks. Early effects on airway inflammation may already be shown after four weeks [16]. However, in clinical trials up to one year Roflumilast showed consistent and sustained improvements in lung function and reduction of exacerbation rates [7]. Based on these clinical trial results the treatment duration of 16 weeks can be considered an acceptable time frame to collect information on the anti-inflammatory effect of Roflumilast and allows sufficient time in between the bronchoscopy procedures and sputum collections.

The trial will not be statistically powered to investigate any clinical outcome measures with regard to the efficacy of COPD treatment, but as mentioned above spirometry measurements will be performed to assess Subject pulmonary function and disease progression. The sample size was calculated based on the available information on the primary variable within the literature and kept as small as possible.

In conclusion the trial is considered as adequately designed to show benefits of Roflumilast treatment to patients in terms of anti-inflammatory effects in the target organ, as well as associated to biomarkers in blood and sputum. Multiple safety aspects are incorporated into the trial protocol to minimize the risk to the participating subjects, while simultaneously having the chance to focus the research efforts on a better understanding of the pathophysiology of COPD within patients in need of new anti-inflammatory treatment options. While Roflumilast is an established product for the reduction of COPD exacerbations, its mechanism in the lungs, particularly its anti-inflammatory activities, are not well understood. Better understanding of its effects on inflammatory cells and the inflammatory cascade may result in a better understanding of which patients would benefit most from a treatment with Roflumilast and which measurable parameters (e.g. cell counts, activities, mediators) might serve as surrogate predictors for the clinical efficaciousness of Roflumilast.

6.3 Premature Termination or Suspension of Study or Investigational Site

6.3.1 Criteria for Premature Termination or Suspension of the Study

The study will be completed as planned unless one or more of the following criteria are satisfied that require temporary suspension or early termination of the study.

- New information or other evaluation regarding the safety or efficacy of the study medication that indicates a change in the known risk/benefit profile for Roflumilast, such that the risk is no longer acceptable for subjects participating in the study.
- Significant violation of Good Clinical Practice (GCP) that compromises the ability to achieve the primary study objectives or compromises subject safety.

6.3.2 Criteria for Premature Termination or Suspension of Investigational Sites

A study site may be terminated prematurely or suspended if the site (including the investigator) is found in significant violation of GCP, protocol, or contractual agreement, is unable to ensure adequate performance of the study, or as otherwise permitted by the contractual agreement.

6.3.3 Procedures for Premature Termination or Suspension of the Study or the Participation of Investigational Site(s)

In the event that the sponsor, an institutional review board (IRB)/independent ethics committee (IEC) or regulatory authority elects to terminate or suspend the study or the participation of an investigational site, a study-specific procedure for early termination or suspension will be provided by the sponsor; the procedure will be followed by applicable investigational sites during the course of termination or study suspension.

7.0 SELECTION AND DISCONTINUATION/WITHDRAWAL OF SUBJECTS

Patients with COPD (GOLD stage II-III, **in Germany stage II only**) are eligible for trial participation.

At least three Subjects per site are required to enable the biopsy sample quality evaluation.

See Section 13.3 for sample size calculation.

All entry criteria, including test results, need to be confirmed prior to randomization or first dose.

7.1 Inclusion Criteria

Subjects meeting the following criteria will be considered for inclusion in the run-in period:

1. Written informed consent obtained according to local regulations before any trial-related activities. A trial-related activity is any procedure that would not have been performed during the routine management of the Subject.
2. History of COPD (according to GOLD 2009) for at least 12 months prior to baseline visit V0 associated with chronic productive cough for at least three months in each of the two years prior to baseline visit V0 (with other causes of productive cough excluded).
3. Outpatients 40-80 years of age.
4. Post-bronchodilator 30% ≤FEV_1 ≤80% predicted.

   (***GERMANY ONLY:*** Post-bronchodilator 50% ≤FEV_1 ≤80% predicted.)
5. Post-bronchodilator FEV_1/FVC ratio ≤70%.
6. Current or former smokers with smoking history ≥20 pack years[2].

7.2 Exclusion Criteria

Subjects meeting any of the following criteria will be excluded from trial enrolment:

Criteria affecting the read-out parameters of the trial:

1. Clinical instability, defined as experiencing a COPD exacerbation six months prior to V0, as indicated by treatment with a systemically administered glucocorticosteroid and/or antibiotics and/or hospitalization.
2. An upper/lower respiratory tract infection e.g. common cold, sinus symptoms, pneumonia, which has not resolved four weeks prior to V0.

[2] Total pack years = No. cigarettes smoked per day/20 x no. years of smoking. For the purpose of the trial a former smoker is defined as a subject who has not smoked for ≥12 months at visit V0. Smoking status will be monitored throughout the trial but not verified by any objective tests.

3. Diagnosis of asthma and/or other relevant lung disease (e.g. history of primary or clinically significant bronchiectases, cystic fibrosis, bronchiolitis, lung resection, lung cancer, interstitial lung disease [e.g. fibrosis, silicosis, sarcoidosis], active tuberculosis) or previous episodes of pneumothorax.
4. Known alpha-1-antitrypsin deficiency.

Criteria within ethical considerations in terms of general health:

5. Duration of oxygen therapy ≥12 h/day.

   (***GERMANY ONLY:*** Any oxygen therapy at home.)
6. History of intubation for COPD or a respiratory failure of any cause in the past year.
7. Hypoxemia defined as oxyhemoglobin concentration <88% when breathing room air measured by pulse oximetry.
8. Formal contraindications to sputum collection or impossibility to obtain a sample of sputum valid for analysis.
9. Clinically relevant abnormal laboratory values suggesting an undiagnosed disease requiring further clinical evaluation (as assessed by the Investigator).
10. Suspicion or diagnosis of a bleeding disorders irrespective of its pathophysiological mechanism: Thrombocytopenia (platelets <50000/mL) and platelet dysfunction (e.g. uraemia), thrombotic or coagulation disorders and bleeding due to abnormal blood vessels (e.g. purpura simplex).
11. Severe psychiatric or neurological disorders.
12. History of depression associated with suicidal ideation or behaviour.
13. Hemodynamic instability (e.g. bradycardia with resting heart rate <60 beats per min, tachycardia with resting heart rate ≥120 beats per min and/or a systolic blood pressure <100 or >180 mmHg).

    13a) (***GERMANY ONLY:*** Hemodynamic instability (e.g. bradycardia with resting heart rate <60 beats per min, tachycardia with resting heart rate ≥100 beats per min and/or systolic blood pressure <100 or >180 mmHg).)
14. History of increased intracranial pressure, superior vena cava obstruction, pulmonary hypertension, aneurysms and arteriovenous malformations within the lungs.
15. Congestive heart failure severity grade IV according to New York Heart Association Functional Classification (NYHA).

    15a) (***POLAND ONLY:*** Congestive heart failure severity grade III and IV according to New York Heart Association Functional Classification (NYHA).)

16. Haemodynamically significant cardiac arrhythmias or heart valve deformations.
17. History of angina pectoris (Class ≥II Canadian Cardiovascular Society Functional Classification of Angina Pectoris Scale) or a myocardial infarction within last 12 months, unless corrected with Percutaneous Transluminal Coronary Angioplasty (PTCA) or Coronary Artery Bypass Surgery (CABG) ≥3 months prior to V0 and asymptomatic since then.
18. X-ray Computed Tomography (CT) or X-ray findings indicating a pulmonary disease other than COPD (e.g. tuberculosis, clinically significant bronchiectases, tumours). CT or X-ray results within 12 months prior to visit V0 are required.

    18a) (***GERMANY ONLY:*** X-ray Computed Tomography (CT) or X-ray findings indicating an anomaly or pulmonary disease other than COPD (e.g. tuberculosis, clinically significant bronchiectases, tumours).)
19. Immunological diseases or known infection with Human Immunodeficiency Virus (HIV).
20. Liver impairment Child-Pugh B/C and/or active viral hepatitis.
21. Severe acute infectious diseases.
22. Any diagnosis of a malignant disease (other than basal or squamous cell carcinoma) within five years before enrolment into the trial.
23. Excessive alcohol intake or drug/substance abuse within the past year.
24. Any history of allergies, suspected hypersensitivity and/or contraindication to local anaesthetics (e.g. xylocaine, lignocaine) and/or sedative agents (e.g. benzodiazepines, propofol, opioids) that would not allow adequate anaesthesia and/or bronchoscopy sedation. Suspected hypersensitivity to the trial treatment (Roflumilast) or ingredients thereof, or any other contraindication for the use thereof.
25. Female Subjects of childbearing potential, not using or not willing to continue using a medically reliable method of contraception for the entire trial duration, such as oral, injectable, or implantable contraceptives, or intrauterine contraceptive devices, unless they are surgically sterilized/hysterectomized or post-menopausal >1 year or who are not using any other method of contraception considered sufficiently reliable by the Investigator in individual cases.
26. Pregnancy, breast feeding, planned oocyte donation or oocyte implantation
27. Planned donation of germ cells, blood, organs or bone marrow during the course of the trial.
28. Participation in another clinical trial (use of investigational product or device) within 30 days preceding the baseline visit V0 or re-entry of Subjects previously enrolled in this trial (for exemption see Section 7.4).

29. Suspected inability to comply with trial procedures (e.g. repeated bronchoscopies, bronchial biopsy and sputum induction procedures, language problems, psychological disorders).
30. Suffering from any concomitant disease that might interfere with trial procedures or evaluations according to the Investigator's judgement.
31. Use of disallowed drugs.
32. Employee at the investigational site, relative or spouse of the Investigator.

7.3 Randomization Criteria

Subjects must meet all the following randomization criteria at V2 to be eligible for randomization into the double-blind treatment period (V2 = randomization visit):

1. No COPD exacerbation between V0 and V2 (as defined by the need for oral or parenteral glucocorticosteroid intake and/or hospitalization).
2. Tablet compliance ≥80% and ≤125%.
3. The admission criteria at inclusion are still met.

Only Subjects who meet all randomization criteria will undergo the first bronchoscopy / bronchial biopsy.

7.4 Re-Enrolment

Re-enrolment of Subjects is only allowed for subjects who failed screening or have to stop the study participation before randomization. After 4 weeks waiting time re-enrolment will be allowed once. However, Subjects who were withdrawn at visit V2 due to an exacerbation (moderate and/or severe exacerbation, and/or treated with antibiotics) between visits V0 and V2 may be re-enrolled after resolution of the exacerbation. If, during the second enrolment, the Subject again experiences such an exacerbation, the Subject must be withdrawn and no further re-enrolment is allowed.

In case a Subject is re-enrolled, the exclusion criteria nos. 1 and 28 do not apply. (see Section 7.2).

After having been randomized the Subject cannot be re-enrolled. For re-enrolled Subjects a new CRF has to be filled in starting at visit V0. A comment specifying that the Subject has been re-enrolled must be given on the screening log and in both CRFs.

7.5 Diet, Fluid, Activity Control

Subjects should not take any food or drink overnight for at least 8 hours prior to returning to the study center for all visits. Subjects will also be asked to avoid strenuous exercise for 8 hours prior to each study visit and to avoid smoking for 4 hours prior to each study visit. For visits where subjects will not undergo blood draws or biopsies, the fasting requirement will only be mandated if clinically indicated as per investigator judgment.

Study medication will be administered once daily in the morning after breakfast with water. On study visit days where the subject is required to fast, study medication will be taken with water only.

7.6 Excluded Medications

The following medications are not allowed throughout the trial and are to be withdrawn prior to or at V0:

1. Inhaled Corticoid Steroids (ICS) or intranasal corticosteroids.
2. Combined formulas of inhaled Long-Acting ß$_2$-Agonists (LABA) and ICS (LABA/ICS).
3. Theophylline and/or derivates (aminophylline, diprophylline) or combinations thereof or any other theophylline containing products.
4. Oral ß$_2$-agonists.
5. Lipoxygenase inhibitors.
6. Leukotriene antagonists.
7. Any combinations of the above mentioned medications.
8. Oral or parenteral anticoagulant therapy: heparin or low molecular weight heparin (LMWH), vitamin K antagonists (e.g. warfarin); factor Xa (e.g. fondaparinux) or thrombin inhibitors (e.g. argatroban).
9. Antiplatelet therapy with thienopyridine (e.g. ticlopidine, clopidogrel, prasugrel) or non-thienopyridine drugs (e.g. cangrelor, ticagrelor).
10. Oral/parenteral glucocorticosteroids or any other immunosuppressive medication (e.g. cyclosporine, methotrexate, TNF alpha receptor inhibitors or antibodies, gold, azathioprine).
11. All available phosphodiesterase 4 (PDE4) inhibitors.

7.7 Allowed Concomitant Medications

1. Rescue medication (any SABA or SAMA) can be used as rescue medication according to the individual needs of a Subject.
2. *Prn* NSAIDs
3. Use of acetylsalicylic acid in doses 75-325 mg for primary or secondary cardiovascular prophylaxis
4. Prophylactic vaccination is allowed throughout the trial
5. Other drugs for the treatment of concurrent diseases are allowed. However, their dosages should be kept constant throughout the trial.

Limited concomitant medication:

1. Therapy consisting of any bronchodilator such as SABA, SAMA, LABA, LAMA starting at least six weeks prior to V0 is allowed. The treatment must remain stable in the course of the whole trial six weeks of single-blind placebo administration (run-in) and 16 weeks of double-blind treatment period.

7.8 Criteria for Discontinuation or Withdrawal of a Subject

The primary reason for discontinuation or withdrawal of the subject from the study or study medication should be recorded in the case report form (CRF) using the following categories. For screen failure subjects, refer to Section 9.1.13.

- Adverse event (AE). The subject has experienced an AE that requires early termination because continued participation imposes an unacceptable risk to the subject's health or the subject is unwilling to continue because of the AE.
- Continued participation in the trial appears to be medically harmful to the Subject (Investigator judgement)
- The Subject wishes to be withdrawn (withdrawal of consent)
- Lost to follow-up
- Closure of investigational site
- Pregnancy or lack of adequate contraception in women of childbearing potential

  Note: If the subject is found to be pregnant, the subject must be withdrawn immediately. The procedure is described in Section 9.1.10.
- Breaking the blind
- Meeting at least one of the following discontinuation criteria:

  1. COPD exacerbation between V0 and V2 (as defined by the need for oral or parenteral glucocorticosteroid intake and/or hospitalization)
  2. A deterioration in pulmonary function that in the judgment of the Investigator compromises a Subject's health

In case of hospitalization, the Investigator should notify the Sponsor within 24 hours (see Section 10.2.2).

A Subject that has been prematurely discontinued, if possible should be called in for a last visit. All procedures listed for VE (visit end, V6) should be completed at this visit. Procedures to be followed in case of premature discontinuation in relation to the point in time during the trial are given in Section 9.3.5. Even if the Subject is not able to attend, the CRF must be completed with all available data and the Drug Accountability Form must be filled in as well. If possible, the Subject must return all trial treatment.

In case of Subjects lost to follow-up, every reasonable effort should be made to contact the Subject to encourage the Subject to continue trial participation as scheduled.

If the Subject withdraws consent, no further evaluations should be performed and no attempts should be made to collect additional data.

Subject recruitment will continue until the planned number of randomized Subjects is reached. All reasons for early withdrawal will be recorded in the CRF.

For follow-up of AEs please refer to Section 10.2.1.

For discontinuation criteria for the trial please refer to Section 6.3.

7.9 Procedures for Discontinuation or Withdrawal of a Subject

The investigator may discontinue a subject's study participation at any time during the study when the subject meets the study termination criteria described in Section 7.8. In addition, a subject may discontinue his or her participation without giving a reason at any time during the study. Should a subject's participation be discontinued, the primary criterion for termination must be recorded by the investigator. In addition, efforts should be made to perform all procedures scheduled for the Early Termination Visit. Discontinued or withdrawn subjects will not be replaced.

8.0 CLINICAL TRIAL MATERIAL MANAGEMENT

This section contains information regarding all medication and materials provided directly by the sponsor, and/or sourced by other means, that are required by the study protocol, including important sections describing the management of clinical trial material.

8.1 Study Medication and Materials

8.1.1 Dosage Form, Manufacturing, Packaging, and Labeling

Study medication refers to Roflumilast 500 µg tablets and matching placebo tablets. Study medication will be packaged in a blinded fashion. The terms "study medication" and "investigational drug" and "sponsor-supplied drug" may be used interchangeably.

All study medication supplied by the Sponsor for this trial will be manufactured, tested and released according to current Good Manufacturing Practice (GMP) guidelines.

The study medication for this trial will be packed and labelled according to current GMP guidelines, Good Clinical Practice (GCP) guidelines and local regulations.

8.1.1.1 Investigational drug

- Roflumilast, 500 µg tablet, once daily, oral administration in the morning after breakfast.
- Placebo tablet, once daily, oral administration in the morning after breakfast.

Roflumilast 500 µg and placebo will be supplied as yellow triangular tablets in wallet cards containing 60 tablets. Each Roflumilast tablet contains 500 µg Roflumilast. Placebo tablets will have an identical appearance.

Each medication kit may have a label with a tear-off part. When the medication is issued to the Subject, if there is a tear-off part, it must be removed and attached to the Subject's Drug Accountability Form included in the CRF.

The investigational sites will be supplied with study medication according to the site's needs depending on the rate of Subject enrolment. Each site will have an individual stock of study medication, which will be resupplied continuously as soon as the amount of study medication will fall below a predefined minimum level. This will be managed by an Interactive Voice Response System (IVRS)/Interactive Web Response System (IWRS).

The study medication will be dispensed at visits V0, V2, and V4. At each dispensing visit, Subjects will be supplied one (V0) or two (V2, V4) wallet cards of study medication (placebo or Roflumilast) each containing 60 tablets (see Table 8.a). Study medication dispensing at visits V2 and V4 allows for a four weeks recovery period in case the Subject experiences an exacerbation (see Section 9.3.4).

Table 8.a Dispensing of Trial Treatment

Visit	No. of weeks since randomization	No. of weeks to next dispensing / returning visit	No. of wallet cards to be dispensed	Number of tablets
V0	-6	6	1	60
V1	-2	-	-	-
V2	0	6	2	120
V3	≤2	-	-	-
V4	6	10	2	120
V5	14	-	-	-
V6	16	-	-	-

8.1.2 Storage

Investigational drug must be kept in an appropriate, limited-access, secure place until it is used or returned to the sponsor or designee for destruction. Study medication must be stored below 30°C, and remain in the original container until dispensed. A daily temperature log of the drug storage area must be maintained every working day.

8.1.3 Dose and Regimen

The approved dose in the EU is Roflumilast 500 µg.

Subjects will be asked to self-administer the trial treatment according to the following regimen:

- Single-blind baseline period (V0-V2): one placebo tablet once daily.
- Double-blind treatment period (V2 [randomization]-VE): one tablet containing placebo or Roflumilast 500 µg once daily.

The tablets must be taken orally in the morning after breakfast with some water. On study visit days where the subject is required to fast, study medication will be taken with water only.

8.1.4 Overdose

An overdose is defined as a known deliberate or accidental administration of investigational drug, to or by a study subject, at a dose above that which is assigned to that individual subject according to the study protocol.

Serious adverse events (SAEs) associated with overdose should be reported according to the procedure outlined in Section 10.2.2, Collection and Reporting of SAEs.

In phase I studies, the following symptoms were observed at an increased rate after a single oral dose of 2,500 µg in seven healthy subjects and one single oral dose of 5,000 µg (ten times the recommended dose) in one healthy subject: headache, gastrointestinal disorders, dizziness, palpitations, light-headedness, clamminess and arterial hypotension. A few serious cases of overdose were identified during marketing of roflumilast for COPD. This limited experience with roflumilast overdose suggests that severe clinical consequences of high doses up to 20 daily doses are not to be expected and observation of subjects rather than risky invasive procedures such as forced gastric emptying is preferable.

In case of overdose it is recommended that the appropriate supportive medical care is provided. Since Roflumilast is highly protein bound, haemodialysis is not likely to be an efficient method of its removal. It is not known, whether Roflumilast is dialysable by peritoneal dialysis.

8.2 Investigational drug Assignment and Dispensing Procedures

The investigator or investigator's designee will access the IVRS/IWRS at Screening to obtain the subject study number and will utilize the IVRS/IWRS to randomize the subject into the study. During this contact, the investigator or designee will provide the necessary subject-identifying information, including the subject number assigned at screening. The medication identification (ID) number of the investigational drug to be dispensed will then be provided by the IVRS/IWRS. If sponsor-supplied drug (Roflumilast, 500 ug tablet, once daily and Placebo tablet, once daily) is lost or damaged, the site can request a replacement from IVRS/IWRS. (Refer to IVRS/IWRS manual provided separately.) If the label includes a tear-off portion, this will be affixed to the CRF. If there is no tear-off portion of the label, the CRF will be completed by hand writing the required information in the space provided on the form. At subsequent drug-dispensing visits, the investigator or designee will again contact the IVRS/IWRS to request additional investigational drug for a subject. The medication ID number of the investigational drug to be dispensed will be provided by the IVRS/IWRS.

In this trial, a 1:1 randomization is employed for both treatments, thus 150 Subjects will be treated with either Roflumilast 500 µg or placebo. Randomization will be stratified by concomitant LABA use, since the DAXAS® pivotal trial pool suggests LABA may be a confounding variable.

8.3 Investigational Drug Blind Maintenance

The investigational drug blind will be maintained using the IVRS/IWRS.

During the single-blind baseline period the Sponsor and Investigator will know that the Subject is receiving placebo. Care should be taken to avoid informing the Subject of the placebo treatment during the baseline period. During the double-blind treatment period and until database freeze, all parties involved in the trial (e.g. Subjects, Investigators, site personnel, clinical research assistants [CRAs], Sponsor) will be blinded.

To maintain the blind, Roflumilast and placebo tablets will be of identical appearance, shape and colour (both yellow, triangular tablets embossed with a 'D') and will have identical labelling and packaging.

8.4 Unblinding Procedure

The investigational drug blind shall not be broken by the investigator unless information concerning the investigational drug is necessary for the medical treatment of the subject. In the event of a medical emergency, if possible, the medical monitor should be contacted before the investigational drug blind is broken to discuss the need for unblinding.

For unblinding a subject, the investigational drug blind can be obtained by the investigator, by accessing the IVRS/IWRS.

The sponsor must be notified as soon as possible if the investigational drug blind is broken. The date, time, and reason the blind is broken must be recorded in the source documents and the same information (except the time) must be recorded on the CRF.

If any site personnel are unblinded, investigational drug must be stopped immediately and the subject must be withdrawn from the study.

The CRA and the sponsor will be notified of each unblinding by the IVRS/IWRS.

8.5 Accountability and Destruction of Sponsor-Supplied Drugs

Drug supplies will be counted and reconciled at the site before being returned to the sponsor or designee.

The investigator or designee must ensure that the sponsor-supplied drug is used in accordance with the protocol and is dispensed only to subjects enrolled in the study. To document appropriate use of sponsor-supplied drug (Roflumilast, 500 µg tablet, and Placebo tablet) the investigator or designee must maintain records of all sponsor-supplied drug delivery to the site, site inventory, dispensation and use by each subject, and return to the sponsor or designee.

Upon receipt of sponsor-supplied drug, the investigator or designee must verify the contents of the shipments against the packing list. The verifier should ensure that the quantity is correct, and the medication is in good condition. If quantity and conditions are acceptable, investigator or designee should acknowledge the receipt of the shipment by recording in IVRS/IWRS. If there are any discrepancies between the packing list versus the actual product received, Takeda must be contacted to resolve the issue. The packing list should be filed in the investigator's essential document file.

The investigator or designee must maintain 100% accountability for all sponsor-supplied drugs received and dispensed during his or her entire participation in the study. Proper drug accountability includes, but is not limited to:

- Frequently verifying that actual inventory matches documented inventory.
- Verifying that all wallet cards used are documented accurately on the log.
- Verifying that required fields are completed accurately and legibly.

If any dispensing errors or discrepancies are discovered, the sponsor must be notified immediately.

The IVRS/IWRS will include all required information as a separate entry for each subject to whom sponsor-supplied drug is dispensed.

The investigator or designee must record the current inventory of all sponsor-supplied drugs (Roflumilast, 500 µg tablet, and Placebo tablet) on a sponsor-approved drug accountability log. The following information will be recorded at a minimum: protocol number and title, name of investigator, site identifier and number, description of sponsor-supplied drugs, expiry date and amount dispensed including initials of the person dispensing the drug, and the date and amount returned to the site by the subject, including the initials of the person receiving the sponsor-supplied drug. The log should include all required information as a separate entry for each subject to whom sponsor-supplied drug is dispensed.

All study drug that was not returned to the site by a subject must be investigated by the site and appropriately documented on the drug accountability log.

Prior to site closure or at appropriate intervals, a representative from the sponsor or its designee will perform sponsor-supplied drug accountability and reconciliation before sponsor-supplied drugs are returned to the sponsor or its designee for destruction. The investigator or designee will retain a copy of the documentation regarding sponsor-supplied drug accountability, return, and/or destruction, and originals will be sent to the sponsor or designee.

9.0 STUDY PLAN

9.1 Study Procedures

The following sections describe the study procedures and data to be collected. For each procedure, subjects are to be assessed by the same investigator or site personnel whenever possible. The Schedule of Study Procedures is located in Appendix A.

9.1.1 Informed Consent Procedure

The requirements of the informed consent are described in Section 15.2.

Informed consent must be obtained prior to the subject entering into the study, and before any protocol-directed procedures are performed.

A unique subject identification number (subject number) will be assigned to each subject by the IVRS system when a subject is screened; this subject number will be used throughout the study.

A separate informed consent will be prepared for the Microbiome Sub-Trial (Section 9.1.15.3), Transcriptom Sub-study (only relevant to the site of Prof Rabe) and the Airway Reflux Sub-study (only relevant to the site of Professor Morice). Sub-study procedures will only be conducted in subjects who have provided consent to these separate sub-study ICFs.

9.1.2 Demographics, Medical History, and Medication History Procedure

Demographic information to be obtained will include date of birth or age (depends on local confidentiality requirement), sex, race as described by the subject, and smoking status of the subject at Screening.

Medical history to be obtained will include determining whether the subject has any significant conditions or diseases relevant to the disease under study that stopped at or prior to signing of informed consent. Ongoing conditions are considered concurrent medical conditions (see Section 9.1.8).

All previous medication administered within the one month prior to visit V0 and all COPD-related medication administered within the 12 months prior to visit V0 must be documented in the CRF. Other relevant previous medication as judged by the Investigator should also be documented in the CRF.

9.1.3 Physical Examination Procedure

A routine physical examination will be done at visits V0, V2, V6, and for Subjects who underwent the second biopsy as planned, at visit V7. Physical examinations will cover eyes, ears, nose, throat, lungs/thorax, heart/cardiovascular system, abdomen, skin and mucosae, nervous system, lymph nodes, musculo-skeletal system, and, if applicable, others. Any new clinically relevant finding compared to visit V0 must be documented as AE in the CRF.

If any clinically relevant finding will be observed at visit V0, these must be documented in the Medical History (MH) section of the CRF.

9.1.4 Weight, Height

Body weight measurements will be performed at each visit from V0 to V7. Measurements should be done to the nearest 0.1 kg after emptying the bladder and with Subjects standing barefoot and wearing light indoor clothing.

Body height will be measured at visit V0 during the run-in period in bare feet, standing Subjects.

9.1.5 Vital Sign Procedure

Vital signs will be measured at each visit and will include heart rate (beats/minute) and blood pressure (mmHg). The Subject should rest for at least 15 minutes prior to measurements. The measurements can be performed either in sitting or supine position of the Subject. The right or left arm may be used. However, the position and the arm used for measurement should be kept constant throughout the trial for an individual Subject.

The Investigator should ensure that each parameter outside the normal range is assessed for clinical significance. Vital signs values beyond the alert limits as defined in Appendix E are considered possibly clinically significant. For any deviation beyond the alert limits detected at any visit after V0, the Investigator has to document the change as an AE in the CRF. In addition, it is at the discretion of the Investigators to document any change or trend over time in vital signs as an AE if they consider the change to be clinically significant, even if the absolute value is within the alert limit or reference range.

The diagnosis of a concomitant disease resulting from measurements of vital signs at visit V0 must be documented in the medical history (MH) section of the CRF. No AE has to be recorded if the parameter deviated from the alert limit at V0 already.

9.1.6 Primary Efficacy Measurement

COPD is a progressive lung disease with inflammatory processes in the lung and bronchi tissue.

This trial will be conducted to investigate the effect of Roflumilast on the characteristic airway inflammation in patients with COPD (GOLD stage II-III, **in Germany stage II only**).

The trial will not be statistically powered to investigate any clinical outcome measures with regard to the effectiveness of COPD treatment; however the results of the pulmonary function tests will be used to better characterize patient population.

9.1.7 Documentation of Concomitant Medications

Concomitant medication is any drug given in addition to the study medication. These may be prescribed by a physician or obtained by the subject over the counter. Concomitant medication is not provided by Takeda. At each study visit, subjects will be asked whether they have taken any medication other than the study medication (used from signing of informed consent through the end of the study), and all medication including vitamin supplements, over-the-counter medications, and oral herbal preparations, must be recorded in the CRF.

9.1.8 Documentation of Concurrent Medical Conditions

Concurrent medical conditions are those significant ongoing conditions or diseases that are present at signing of informed consent. This includes clinically significant laboratory, electrocardiogram (ECG), chest X-ray, CT scan or physical examination abnormalities noted at Screening and/or prior to Randomisation (V2). The condition (ie, diagnosis) should be described.

9.1.9 Procedures for Clinical Laboratory Samples

The central laboratory will provide the sites with appropriate material and a manual before the trial starts.

All samples will be collected in accordance with acceptable laboratory procedures. The maximum volume of blood at any single visit is approximately 18,5 mL, and the approximate total volume of blood for the study is 58 mL for all scheduled visits. Details of these procedures and required safety monitoring will be given in the laboratory manual.

Table 9.a Clinical Laboratory Tests

Hematology	**Serum Chemistry**	**Urinalysis**
Haemoglobin	Glucose	Glucose
Haematocrit,	Creatinine	Protein
Erythrocytes	Total bilirubin (in case of values above the normal range, direct and indirect bilirubin will be determined),	Blood
Thrombocytes,		
Leukocytes	Alkaline phosphatase (AP)	
Differential white blood cell (WBC) count	Aspartate aminotransferase (AST)	
	Alanine aminotransferase (ALT)	
	Gamma-Glutamyl transferase (GGT)	
	Creatine kinase (CK)	
	Sodium	
	Potassium	
	Total calcium	
	Cholesterol (total, low density lipoprotein, high density lipoprotein)	
	Triglyceride	
Other:		
Serum	**Urine**	
Female subjects of childbearing potential: human chorionic gonadotropin (hCG) for pregnancy	Female subjects of childbearing potential: hCG for pregnancy	

The central laboratory will perform laboratory tests for hematology, serum chemistries, and urinalysis.

The results of laboratory tests will be returned to the investigator, who is responsible for reviewing and filing these results.

The investigator will maintain a copy of the laboratory accreditation and the reference ranges for the laboratory used.

Analyses will be done by a central laboratory. Blood samples will be handled and stored according to the instructions provided by the central laboratory. All laboratory samples have to be clearly and fully labelled according to the Central Laboratory Manual. The laboratory reports received by fax from the central laboratory will be reviewed, signed and dated by the Investigator and attached to the CRF (including corrected reports).

Blood must be drawn under fasting conditions (8 hours).

In case of creatine kinase (CK) levels above the Sponsor defined alert value (> 3x upper limit of normal range, see Appendix E), the Investigator should ensure that creatine kinase-MB (CK-MB) is determined and the reason for the elevated levels must be explained. Any indication of possible cardiac events should be followed up appropriately by the Investigator.

The diagnosis of a concomitant disease resulting from laboratory parameters at visit V0 must be documented in the medical history (MH) section of the CRF. Clinically relevant abnormal deviations of laboratory parameters (i.e. values suggesting an unknown disease and requiring further clinical evaluation) revealed at baseline visit V0 are not considered to be AEs. If necessary, retesting must be performed at visit V1 and exclusion criteria carefully evaluated.

Laboratory values exceeding the Sponsor-predefined “alert values” (see Appendix E) occurring after visit V0 must be documented as AEs. Additionally, any clinically significant change, as judged by the Investigator, must be reported as AE.

In case of any value being reported as an AE at visit last visit (V6 or V7), a follow-up including further laboratory investigations must be performed.

Blood samples must be collected, prepared, and arranged for transport according to the instructions provided by the central laboratory.

If laboratory results do not appear reliable, e.g. due to haemolysis, a re-investigation is necessary.

9.1.9.1 Urine Analysis

Urine analysis will be performed at visit V0.

Urine analysis with dipstick has to be performed at the site according to the manufacturer's instruction. Dipsticks are provided with the laboratory material.

The following parameters will be determined: glucose, protein, and blood. The diagnosis of a concomitant disease resulting from urine analysis has to be documented in the MH section of the CRF. The Subject must be excluded from the trial if a forbidden concomitant disease is diagnosed.

If the reliability of the laboratory results is doubtful, an immediate re-investigation of the laboratory variables is recommended.

9.1.9.2 Clotting Time and Platelet Count

Blood sampling will be performed at visits V0, V1, and V5. Analyses of the clotting screen (PT, APTT, TT, INR and platelet count) will be done by the Central Safety Laboratory. Blood samples will be handled and stored according to the instructions provided by the Central Safety Laboratory. All laboratory samples have to be clearly and fully labelled according to the Central Laboratory Manual. The laboratory reports received by fax from the Central Safety Laboratory will be reviewed, signed and dated by the Investigator and attached to the CRF (including corrected reports).

9.1.10 Contraception and Pregnancy Avoidance Procedure

From signing of informed consent, throughout the duration of the study, and for 30 days after last dose of study medication, female subjects of childbearing potential* who are sexually active with a nonsterilized male partner** must use adequate contraception. In addition they must be advised not to donate ova during this period.

*Females NOT of childbearing potential are defined as those who have been surgically sterilized (hysterectomy, bilateral oophorectomy or tubal ligation) or who are postmenopausal (eg, defined as at least greater than 12 months after last menstruation, confirmed before any study medication is implemented).

**Sterilized males should be at least 1 year postvasectomy and have confirmed that they have obtained documentation of the absence of sperm in the ejaculate.

An acceptable method of contraception is defined as one that has no higher than a 1% failure rate. The only acceptable methods of contraception are:

Barrier methods (each time the subject has intercourse):

- Male condom PLUS spermicide.
- Cap (plus spermicidal cream or jelly) PLUS male condom and spermicide.
- Diaphragm (plus spermicidal cream or jelly) PLUS male condom and spermicide.

Intrauterine devices (IUDs):

- Copper T PLUS condom or spermicide.
- Progesterone T PLUS condom or spermicide.

Hormonal contraceptives:

- Implants.
- Hormone shot/injection.
- Combined pill.
- Minipill.
- Patch.
- Vaginal ring PLUS male condom and spermicide.

Subjects will be provided with information on acceptable methods of contraception as part of the subject informed consent process and will be asked to sign a consent form stating that they understand the requirements for avoidance of pregnancy, donation of ova, during the course of the study.

During the course of the study, regular serum human chorionic gonadotropin (hCG) pregnancy tests will be performed only for women of childbearing potential and subjects will receive continued guidance with respect to the avoidance of pregnancy as part of the study procedures (Appendix A). Subjects must have a negative urine hCG pregnancy test at V2 prior to receiving first dose of investigational drug.

9.1.11 Pregnancy

If any subject is found to be pregnant during the study she should be withdrawn and any sponsor-supplied drug

- Roflumilast, 500 µg tablet, once daily
- Placebo tablet, once daily,

should be immediately discontinued. If the pregnancy occurs during administration of active study medication, eg, after Visit V2 or within 30 days of the last dose of active study medication, the pregnancy should be reported immediately, using a pregnancy notification form, to the contact listed in Section 1.0.

Should the pregnancy occur during or after administration of blinded drug, the investigator must inform the subject of their right to receive treatment information. If the subject chooses to receive unblinded treatment information, the individual blind should be broken by the investigator. Subjects randomized to placebo need not be followed.

If the female subject and/or female partner of a male subject agrees to the primary care physician being informed, the investigator should notify the primary care physician that the subject/female partner of the subject was participating in a clinical study at the time she became pregnant and provide details of treatment the subject received (blinded or unblinded, as applicable).

All pregnancies in subjects on active study drug including comparator will be followed up to final outcome, using the pregnancy form. Pregnancies will remain blinded to the study team. The outcome, including any premature termination, must be reported to the sponsor. An evaluation after the birth of the child will also be conducted.

9.1.12 ECG Procedure

A 12-lead ECG has to be done at visit V0. An ECG will be also done and repeated, if the Investigator considers it necessary. The Subject should rest for at least 15 minutes prior to the recording, and should be in a supine position during the recording. Resting ECG should be performed before any pulmonary function testing.

The Investigator will be responsible for interpretation of the ECG to ensure Subject safety. The ECG printout will be reviewed, interpretation should be noted, and the printout will be signed and dated by the Investigator and will be filed at the investigational site as source data and a copy will be filed in the CRF.

If any clinically relevant finding will be observed at visit V0, these must be documented in the medical history (MH) section of the CRF.

9.1.13 Documentation of Screen Failure

Investigators must account for all subjects who sign informed consent.

If the subject is found to be not eligible at the Screening Visit, the investigator should complete the appropriate CRFs. The IVRS/IWRS should be contacted as a notification of screen failure.

For re-enrolled Subjects a new CRF has to be filled in starting at Visit V0. A comment specifying that the Subject has been re-enrolled must be given on the screening log and in both CRFs.

The primary reason for screen failure is recorded in the CRF.

Subject numbers assigned to subjects who fail screening should not be reused.

If the subject has begun the single-blind placebo run-in study medication and they are found to not be eligible for randomization, they are considered a randomization failure. The investigator or designee should complete the appropriate CRFs and register the subject as a randomization failure in the IVRS/IWRS.

9.1.14 Documentation of Randomization

Only subjects who meet all of the inclusion criteria and none of the exclusion criteria are eligible for randomization into the treatment phase.

If the subject is found to be not eligible for randomization, the investigator should record the primary reason for failure on the applicable CRF.

9.1.15 Bronchoscopy

Bronchoscopy will be performed at visit V2 (randomization visit) and at visit V6 (subsequent to 16 weeks of treatment) in line with the American Thoracic Society guidelines and Endobronchial Biopsy Workshop [13, 18], a slightly modified, protocol of O'Shaughnessy et al. [1997] [19], and according to clinical standards of care at the site.

Endobronchial biopsies will be taken from each the lobar and sub segmental carinae. At each bronchoscopy session, in order to take into account within-Subject variability, 2-3 biopsies will be taken from the lobar bronchus and 2-3 from the sub-segmental airways. Additionally the left and right lobes will be alternated per Subject, but all biopsies will be harvested from one lung at a given session. Cup forceps will be provided by the Sponsor for the sole use in the course of this trial to maintain the specimen quality. However, other suitable forceps may also be used.

Although bronchoscopy is considered to be safe procedures in patients with COPD [20], Subjects will be closely monitored after bronchoscopy for at least 2 h. Subjects will only be discharged when the effects of sedation and local anaesthesia disappear and no hypoxemia, while breathing room air will be observed. All Subjects will be given a 24 h emergency contact number.

Additionally a safety visit will be performed within two weeks, during which the respective Investigator will see the Subjects, after each bronchoscopy session.

Further details are described in the Bronchoscopy Manual (Appendix F).

9.1.15.1 Biopsy Sample Processing and Cell Quantification

Biopsies will gently be extracted from the forceps and sent to the site laboratory for further processing (fixation and paraffin wax-embedding). Immunostaining and quantification of inflammatory cells will be performed according to standard procedures by a central pathology laboratory. [13, 19, 21, 22].

Inflammatory cells will be identified using specific monoclonal antibodies. For each antibody, the total number of positively stained cells will be counted to a depth of 100 μm below the epithelial basement membrane (i.e. the area of subepithelium excluding muscle, gland and large vessels) using a computerized image analysis [22]. Slides will be evaluated centrally by a reader blinded to the treatment arm with counts obtained from all 4-6 biopsies for each biopsy occasion, where possible. Following cells are to be counted in the biopsied material [cell/mm^2]: CD8+, CD68+, neutrophils, CD4+, CD45+. In addition CD8+ and CD68+ will also be counted in bronchial epithelium.

Additional cells may potentially be subject to exploratory analysis in biopsied material after the completion of the current trial.

Further details are described in the Biopsy Laboratory Manual (Appendix G).

9.1.15.2 Internal Process to Assure Biopsy Sample Quality

To ensure adequate quality and consistency between Investigators at sites, training will be conducted on all aspects of biopsy material collection, handling and processing.

The quality of biopsied material will be validated for each bronchoscopist at the site. Sites will be requested to provide pseudonymized tissue samples (2-3 lobar bronchus and 2-3 sub-segmental airways specimens per Subject) harvested from the first three enrolled Subjects, which are expected to be recruited within three months from site initiation. Half a section per biopsy dyed with haematoxylin eosin staining and antigen preservation assessment will be used to determine the viability and quality of biopsied tissue. In order to be considered a good quality sample the biopsied tissue area must be of ≥0.3 mm^2 containing ≥1 mm baseline and be ≥100 micron deep to enable good cell counts. Mucosa should be in good shape (as intact as possible) and viable antigen presentation must be established. The actual cell counting will not be performed during this process. Further details will be provided in the Bronchoscopy Manual (Appendix F) and in the Biopsy Laboratory Manual (Appendix G).

Only after the biopsy samples are deemed to be of sufficiently good quality by the central reader(s), will the respective bronchoscopist at the site be allowed to randomize further Subjects into the trial. The limit of 3 patients will be extended by another 3 patients in case the quality of one or more of the first samples is not sufficient and to give the site a chance to improve the

sampling technique. In very exceptional cases and if the reason for failure is outside of the control of the site a further extension by additional 2 patients will be allowed.

For avoidance of doubt, all available data will be included in the analysis.

9.1.15.3 Brushing Specimens from the Right Middle Lobe for Microbiome Assessments (Sub-Trial in selected Subjects only)

Participation in the Microbiome Sub-Trial is optional, involving only those subjects who voluntarily opt to provide their consent to participate in the Sub-Trial. In these selected Subjects three protected brush specimens will be collected during the bronchoscopy procedures at visits V2 and V6. If performed, these specimens should be collected after the bronchial biopsy procedure in the right middle lobe bronchus. In addition, to examine whether microbiota from bronchoscopic samples is from contamination from the fibreoptic bronchoscope or the mouth, a sample of a saline mouthwash and a saline sample drawn through the suction channel of the bronchoscope will be obtained before each bronchschopic procedure.

The specimens will be shipped in a frozen state via PPD's Global Central Labs to , University of Ann Arbor, Michigan, U.S.A. for further evaluation.

The specimens will be used to evaluate longitudinal changes in COPD airways microbiota in placebo-treated Subjects and to define the effect of Roflumilast therapy on the airway microbiome. An additional Subject Informed Consent will be collected prior to participation of any Subjects in this sub-trial. Exact details and procedures are described in Appendix I. All analyses will be specified in a separate Statistical Analysis Plan and the results will be reported in a separate Clinical Trial Report.

9.1.16 Spirometry/Pulmonary Function Test

Spirometry for pulmonary function testing will be performed according to the recommendations of the American Thoracic Society – European Respiratory Society (ATS/ERS) consensus guidelines on pulmonary function testing [23].

Sites will use their own devices, performing maintenance and calibration of instruments according to their usual standards of practice. An originally signed and dated printout of lung function measurements has to be stored in the Subject file on site.

The following parameters will be recorded in the CRF:

- FEV_1: Forced expiratory volume in the first second (absolute and %-predicted values);
- FVC: Forced vital capacity (expiratory) (absolute values);
- FEV_1/FVC: Ratio of forced expiratory volume after one second to forced vital capacity.

Generally, only post-bronchodilator values are assessed.

FEV_1 and FVC will be chosen as the largest value from the different efforts. These values may come from different test curves. FEV_1/FVC will be calculated from the largest FEV_1 and largest FVC.

- The pulmonary function tests should always be performed in the morning hours between 8 am and 12 am and ± 4 hours versus the measurement at visit V0.
- The Subject should have been at rest 15 to 30 minutes prior to each pulmonary function test.
- All measurements are to be made with the Subjects seated upright.
- Measurements are to be made with nose clips.
- The post-bronchodilator measurements must be performed 30 minutes (± 15 minutes) after four inhalations of 100 µg salbutamol (in total 400 µg) from an MDI with a spacer.
- The same equipment will be used throughout the trial and, whenever possible, the same person should perform the measurements.

9.1.17 Sputum Induction

Sputum will be induced and collected at visits V1 (-2 weeks), V4 and V5 (subsequent to six and 14 weeks of double-blind treatment respectively). Sputum samples will be collected and initially processed at the investigational sites. Further details on sputum induction procedures and sputum sample processing are provided in the Sputum Laboratory Manual 'Manual Sputum Induction and Processing in COPD studies (Differential Cell Counts and Biomarkers)' (see Appendix H).

To ensure adequate quality and consistency of sputum samples between investigational sites, centralized hands-on training sessions in sputum collection, handling and processing will be provided by the Sputum Laboratory.

The quality of sputum samples will be assessed on an ongoing basis. In case of major concerns by the central reader(s) at the Sputum Laboratory about the quality of the sputum samples further procedural steps will be discussed with the Sponsor.

Further operative details are defined in the Sputum Laboratory Manual (see Appendix H) and in the Central Laboratory Manual.

9.1.17.1 Total and Differential Cell Counts in Induced Sputum

The following total and differential cell counts in induced sputum samples will be performed at the Sputum Laboratory:

- Total and differential cell counts in induced sputum (absolute [cells/mL] and percentage [%]):
  1) Neutrophils
  2) Macrophages
  3) Eosinophils

4) Lymphocytes

9.1.17.2 Measurement of Variables Describing Concentration of Inflammatory Biomarkers in Sputum Specimens

Sputum collection for measurement of inflammatory biomarkers will be performed at visits V1 (-2 weeks), V4 and V5 (subsequent to six and 14 weeks of double-blind treatment respectively). Sputum supernatant samples will be handled and stored according to the instructions provided in the Sputum Laboratory Manual (see Appendix H). All laboratory samples have to be clearly and fully labelled according to the Central Laboratory Manual.

Sputum samples will be analysed by the Biomarker Laboratory using the 46-biomarker Multi-Analyte Profiling (MAP) technology (Human InflammationMAP® v.1, Rules-Based Medicine, Inc., Austin, TX, U.S.A.).

The parameters Alpha-2 Macroglobulin, Interleukin-8 (IL-8), Monocyte Chemotactic Protein-1 (MCP-1), Matrix Metalloproteinase Type 9 (MMP-9), Tissue Inhibitor of Metalloproteinase (TIMP) and Vascular Endothelial Growth Factor (VEGF) were described in the literature as disease specific sputum marker for COPD patients [15, 24]. Within this trial a potential pharmacological effect of Roflumilast on these biomarkers of primary interest will be evaluated.

The Multi-Analyte Profiling (MAP) technology (Human InflammationMAP® v.1, Rules-Based Medicine, Inc., Austin, TX, U.S.A.) profile contains quantitative, multiplexed immunoassays of the above mentioned biomarkers of primary interest and in addition 40 further biomarkers attributed to an inflammatory process, summing up to 46 biomarkers overall. All biomarkers of the profile will be analysed in the same pre-specified manner with focus on the above stated biomarkers of primary interest and the remainder of the biomarkers for explorative reasons.

The **Multi-Analyte Profile (MAP)** Human InflammationMAP® v.1 provides the following biomarkers (*biomarkers of primary interest italicized*):

> Alpha-1 Antitrypsin, *Alpha-2 Macroglobulin*, Beta-2 Microglobulin, Brain-Derived Neurotrophic Factor, C Reactive Protein, Complement 3, Eotaxin, Factor VII, Ferritin, Fibrinogen, GM-CSF, Haptoglobin, Intercellular Adhesion Molecule-1, Interferon gamma, Interleukin-1 alpha, Interleukin-1 beta, Interleukin-1 receptor antagonist, Interleukin-2, Interleukin-3, Interleukin-4, Interleukin-5, Interleukin-6, Interleukin-7, *Interleukin-8*, Interleukin-10, Interleukin-12 p40, Interleukin-12 p70, Interleukin-15, Interleukin-17, Interleukin-23, Matrix metalloproteinase type 2, Matrix metalloproteinase type 3, *Matrix metalloproteinase type 9*, Macrophage Inhibitory Protein-1 alpha, Macrophage Inhibitory Protein-1 beta, *Monocyte Chemotactic Protein-1*, RANTES, Stem Cell Factor, *Tissue Inhibitor of Metalloproteinase*, Tumor Necrosis Factor alpha, Tumor Necrosis Factor beta, Tumor Necrosis Factor receptor alpha 2, Vascular Cellular Adhesion Molecule type 1, *Vascular Endothelial Growth Factor*, von Willebrand's Factor, Vitamin D Binding Protein

Database print-outs of the analysis data will be provided to the Investigator in a timely manner subsequent to data unblinding and no later than trial closeout and have to be filed in the Investigator's file together with the CRF data of the respective Subject.

9.1.18 Inflammatory Biomarkers in Blood Serum

9.1.18.1 Blood Withdrawal and Sample Handling Methods

Blood serum collection for measurement of inflammatory biomarkers will be performed at visits V1 (-2 weeks), V4 and V5 (subsequent to six and 14 weeks of double-blind treatment respectively).

Blood withdrawal for the measurement of inflammatory biomarkers will be performed at approximately the same daytime (± 2 hours) but at 10:00 am at the latest at each respective visit.

Further details are described in the Central Laboratory Manual.

9.1.18.2 Measurement of Variables Describing Concentration of Inflammatory Biomarkers and metabolites in Blood Serum

The following inflammatory biomarkers will be measured in the collected blood serum:

1. Inflammatory mediators
   (Human InflammationMAP® v.1, RBM)

Biomarker analyses will be performed by the Biomarker Laboratory. Exact procedures how to handle blood drawing, blood processing, storing and shipment are described in Section 9.4 and in the Central Laboratory Manual.

Database print-outs of the analysis data will be provided to the Investigator in a timely manner subsequent to data unblinding and no later than trial closeout and have to be filed in the Investigator's file together with the CRF data of the respective Subject.

9.1.18.3 Measurement of a panel of different metabolites in blood serum

The metabolomics analysis of the blood samples will be done by Metanomics Health GmbH About 2000 different metabolites (metabolites are the intermediates and products of e.g. protein, fat, vitamin and carbohydrate metabolism) will be analysed in the collected blood serum samples.

A panel of about 2000 different metabolites will be analysed in the blood backup samples, if not used for reanalysis of inflammatory biomarkers.

The data will be analysed for explorative reasons. The metabolomics analyses will be described in a separate statistical analysis plan and finally the data in a separate report. It will be not part of the main SAP or main CSR.

9.2 Monitoring Subject Treatment Compliance

The Investigator must ask the Subject to return excess trial treatment as well as all packing materials (including empty wallet cards) at visits V2, V4, and V6 for trial treatment accountability.

If a subject is persistently noncompliant with the study medication

- Roflumilast, 500 µg tablet, once daily
- Placebo tablet, once daily

it may be appropriate to withdraw the subject from the study. All subjects should be reinstructed about the dosing requirement during study contacts. The authorized study personnel conducting the re-education must document the process in the subject source records.

Compliance will be calculated as follows:

$$\% \text{ compliance} = \frac{\text{Number of tablets taken}}{\text{Number of days in period}} \times 100$$

For the number of days in a period, V_x to V_{x+1} the V_x visit day will be included, the V_{x+1} visit excluded e.g. for the V0 to V2 period the V0 visit day will be included, the V2 visit day excluded.

Compliance must be calculated and recorded at visit V2 for the purpose of randomization: for randomization compliance must be ≥80% and ≤125% during the six-week baseline period (see randomization criteria, Section 7.3).

Compliance for the double-blind treatment period will be calculated after database lock and must be ≥70% and ≤143% during the 16-week treatment period.

9.3 Schedule of Observations and Procedures

Subjects will be given a patient card at visit V0 stating the Subject's name, that the Subject is participating in a clinical trial, and the name, address and telephone number of the respective Investigator. The Subject must be advised to carry this identification card along with him/her throughout the entire trial.

Subjects should be advised to bring to the specified visits the trial treatment (including empty blisters from tablets, and cartons) and, if applicable, a reading aid (e.g. glasses).

The Subject will rest quietly for at least 15 min before any blood for laboratory assessments is drawn (if applicable) and/or any pulmonary function test (see Section 9.1.16) is performed, vital signs are measured, or ECG is performed. Throughout the course of the trial subjects will need to adhere to the following regulations prior to each trial visit:

- **4h:** Avoid smoking
- **8h:** Maintain fasting (refrain from food and drink) and avoid strenuous exercise

At each visit Subjects should be advised that one tablet of trial treatment once daily should be self-administered in the morning. Subjects will be permitted to take medication with water during this period if this is considered acceptable in the clinical judgement of the investigator.. For visits where subjects will not undergo blood draws or biopsies, the fasting requirement will only be mandated if clinically indicated as per investigator judgement.

At each visit the Subjects will be asked to return to the investigational site according to the schedule listed in Appendix A for the following visit. However, the Subjects should also be instructed to contact the investigational site at any time during the entire trial if they experience a pronounced deterioration of their disease. Suitable arrangements for emergency contact by the Subject with the Investigator will be organized. The Subject must be advised to inform the Investigator in case of emergency.

9.3.1 Screening / Visit 0 (V0)

Subjects for whom written informed consent has been obtained (see Section 9.1.1) will undergo the following assessments or procedures:

- Demographic data (e.g. race, sex, date of birth)
- Medical History and concomitant diseases (all relevant previous illnesses, which may or may not be still ongoing at V0, are to be documented)
- COPD history and history of COPD exacerbations
- Check of inclusion and exclusion criteria (see Sections 7.1 and 7.2)
- Previous and concomitant medications (see Sections 7.6, 7.7, 9.1.2)
- Substance use (smoking status)
- Physical examination (see Sections 9.1.3)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight and height) (see Sections 9.1.4, 9.1.5)
- Standard laboratory (including urine analysis and blood pregnancy test for females of childbearing potential, see Section 9.1.9)
- Clotting time and platelet count (see Section 9.1.9.2)
- Chest X-ray or CT-scan (if not performed within the last three months – results must be available)
  - ***GERMANY ONLY:*** Every patient should have a historical X-ray or CT scan available to clarify the exclusion criteria #18.
- 12-lead ECG (see Section 9.1.12)
- Pulmonary function test (see Section 9.1.16)

For Subjects who meet the inclusion criteria:

- Withdrawal of disallowed COPD medication (see Section 7.6)
- Dispense patient card

- The adverse event monitoring will be explained (see Section 10.2.1)
- Call IVRS and dispense trial treatment

Further procedures

The Subject will be asked to return to the investigational site for visit V1 four weeks after visit V0.

9.3.2 Run-in / Visit V1 (4 weeks after baseline visit V0)

The following assessments or procedures will be performed:

- Concomitant medications (see Sections 7.6, 7.7, 9.1.7)
- Substance use (smoking status)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight) (see Section 9.1.4, 9.1.5)
- Standard laboratory if deemed necessary (see Section 9.1.9). Pregnancy test optional.
- Urine pregnancy test (see Section 9.1.10)
- Clotting time and platelet count (see Section 9.1.9.2)
- Blood sampling for assessment of inflammatory biomarkers and metabolomics in blood serum (see Section 9.1.18)
- Sputum induction and sputum sample processing (see Section 9.1.17)
- Adverse event assessment

Further procedures

The Subject will be asked to return to the investigational site for visit V2 two weeks after visit V1.

9.3.3 Randomization / Visit V2 (6 weeks after baseline visit V0)

After baseline visit V0 the Subject will return to the investigational site after six weeks for visit V2. Note that the time period V2 (= randomization) to V0 has to be six weeks. The Subject will return all used and unused trial treatment for a compliance check. The following assessments or procedures will be performed:

- Concomitant medications (see Sections 7.6, 7.7, 9.1.7)
- Substance use (smoking status)
- Physical examination (see Section 9.1.3)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight) (see Sections 9.1.4, 9.1.5)
- Urine pregnancy test (see Section 9.1.10)

- Pulse oximetry measurement during bronchoscopy/bronchial biopsy
- Pulmonary function test (see Section 9.1.16)
- Adverse event assessment
- Return of trial treatment
- Tablet compliance check (see Section 9.2)[3]
- Check of randomization criteria (see Section 7.3)
- Bronchoscopy with bronchial biopsy (see Section 9.1.15)

Note:

In selected Subjects three protected brush specimen from the right middle lobe bronchus will be collected. This is described in Appendix I (see also Section 9.1.15.3).

For Subjects who meet randomization criteria:

- Randomization
- Dispense of trial treatment (Subjects will start taking double-blind trial treatment)

Subjects not fulfilling all randomization criteria after the six-week run-in period must be excluded from the trial.

Further procedures

Subjects will be asked to return to the investigational site for visit V3 ≤2 weeks after visit V2.

9.3.4 Treatment Phase

Each visit during the treatment period should be performed **at nearly the same time as at the randomization visit (V2)**, if possible.

In case a Subject develops the exacerbation during the double-blind treatment period (V2-V6), the Subject should be allowed to continue trial participation. However, sputum induction and bronchial biopsy must be postponed for up to four weeks until the Subject returns to clinically stable condition according to the Investigator's judgement.

9.3.4.1 Visit V3 (≤2 weeks after randomization visit V2)

After randomization visit V2 the Subject will return to the investigational site for visit V3 after ≤2 weeks to get information about the Subject's well being after the bronchoscopy. The following assessments or procedures will be performed:

[3] Tablet compliance calculation by the investigational site (see Section 9.2) to check for randomization criterion no. 2 (see Section 7.3)

- Concomitant medications (see Sections 7.6, 7.7, 9.1.7)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight) (see Sections 9.1.4, 9.1.5)
- Adverse event assessment

Subjects will be asked to return to the investigational site for visit V4 six weeks after randomization visit V2.

9.3.4.2 Visits V4 (6 weeks after randomization visit V2):

After randomization visit V2, the Subject must return to the investigational site after six weeks for the following assessments and procedures:

- Concomitant medications (see Sections 7.6, 7.7, 9.1.7)
- Substance use (smoking status)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight) (see Sections 9.1.4, 9.1.5)
- Urine pregnancy test (see Section 9.1.10)
- Blood sampling for assessment of inflammatory biomarkers and metabolomics in blood serum (see Section 9.1.18)
- Sputum induction and sputum sample processing (see Section 9.1.17)
- Adverse event assessment
- Return of trial treatment
- Tablet compliance check (see Section 9.2)
- Dispense of trial treatment

9.3.4.3 Visits V5 (14 weeks after randomization visit V2):

After randomization visit V2, the Subject must return to the investigational site after 14 weeks for the following assessments and procedures:

- Concomitant medications (see Sections 7.6, 7.7, 9.1.7)
- Substance use (smoking status)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight) (see Sections 9.1.4 9.1.5)
- Urine pregnancy test (see Section 9.1.10)
- Clotting time and platelet count (see Section 9.1.9.2)

- Blood sampling for assessment of inflammatory biomarkers and metabolomics in blood serum (see Section 9.1.18)
- Sputum induction and sputum sample processing (see Section 9.1.17)
- Adverse event assessment

9.3.4.4 Visits V6 (16 weeks after randomization visit V2):

After randomization visit V2, the Subject must return to the investigational site after 16 weeks for the following assessments and procedures:

- Concomitant medications (see Sections 7.6, 7.7, 9.1.7)
- Substance use (smoking status)
- Physical examination (see Section 9.1.3)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight) (see Sections 9.1.4, 9.1.5)
- Standard laboratory (including blood pregnancy test for females of childbearing potential, see Sections 9.1.9, 9.1.10)
- Bronchoscopy with bronchial biopsy (see Section 9.1.15)

  Note:

  In selected Subjects three protected brush specimen from the right middle lobe bronchus will be collected. This is described in Appendix I (see also Section 9.1.15.3).
- Pulse oximetry measurement during bronchoscopy/bronchial biopsy
- Pulmonary function test (see Section 9.1.16)
- Adverse event assessment
- Return of trial treatment
- Tablet compliance check (see Section 9.2)
- Termination of the double-blind treatment phase of the trial

9.3.5 Final Visit or Early Termination

For all **randomized** Subjects who complete the trial as scheduled, the assessments described for V6 must be performed at trial completion. If premature discontinuation after V3 occurs, the following will apply:

- All assessments described for visit V6 must be performed with exception for the bronchoscopy with bronchial biopsy and their related procedures (the bronchoscopy with bronchial biopsy

and their related procedures can only be done upon the Subject's agreement) and the CRF Disposition Page must be completed.

The Disposition Page of the CRF must be completed for all randomized and non-randomized Subjects, terminating the trial (either as scheduled or prematurely). For Subjects terminating prematurely the reason for the premature termination has to be documented. The confirmation of the clinical Investigator, that the Subject has been treated under his/her supervision and data are correctly recorded will be documented in this Disposition CRF Page.

After terminating the trial (after VE) Subjects will be treated according to their medical needs.

9.3.6 Follow-up / Visit V7 (≤2 weeks after visit V6)

For all randomized Subjects who completed the trial as scheduled and the final bronchial biopsy was performed at V6, a follow-up visit (V7) will be performed within two weeks after V6 to receive information about the Subject's well being after the bronchoscopy.

The following assessments or procedures will be performed:

- Concomitant medications (see Sections 7.6, 7.7, 9.1.7)
- Physical examination (see Section 9.1.3)
- Vital signs (e.g. heart rate, blood pressure) and body measurements (body weight and height) (see Sections 9.1.4, 9.1.5)
- Adverse event assessment

9.3.7 Post Study Care

As Roflumilast is registered as DAXAS® and is marketed in several countries, Subjects may receive the medication after trial completion (V6). It is at the discretion of the respective Investigator to prescribe DAXAS® in countries where it is marketed. If the drug is not marketed in the respective country, no further treatment can be granted by the Sponsor.

The blinding of Subjects will be kept in any case until database freeze, unless unblinded according to Section 8.4.

9.4 Biological Sample Retention and Destruction

PPD's global central laboratory will supply the investigational site with the necessary equipment for storage, labelling and shipment of the samples.

Paraffin embedded biopsies, prepared as described in the Biopsy Laboratory Manual (Appendix G), can be stored and shipped at room temperature. Labelling and shipment are described in the Central Laboratory Manual.

The Investigator is responsible for the proper storage of biological samples (i.e. blood samples for standard safety laboratory analyses, blood samples for inflammatory biomarker analyses, sputum materials) at approximately -20°C or below (preferably -80°C) until sample shipment.

All biological samples have to be clearly and fully labelled according to the Central Laboratory Manual. Biological samples should be sorted by Subject and visit such that all the samples from a given Subject are readily accessible. A sample inventory list of all biological samples must be included in each sample shipment. Packing and shipment of the biological samples must be in accordance with the current guidelines.

Further details for storage, labelling and shipment of (a) biopsy paraffin blocks, (b) standard safety laboratory blood samples, (c) sputum cell count samples, (d) sputum biomarker samples, and (e) biomarker blood samples are described in the Central Laboratory Manual.

PPD will analyse the standard safety laboratory parameters immediately, while the serum and sputum supernatant samples for inflammatory biomarkers analyses will be stored at -80°C at PPD's global central laboratory. On Sponsor's request the stored samples will be sent on dry ice from PPD's global central laboratory to the Biomarker Laboratory (RBM, Austin, Texas) for analysis. PPD will be advised of the shipping details. Frequency of shipments will be discussed between the Sponsor and PPD by case situation.

Aliquots of sputum and tissue samples of the biopsy material will be collected and stored for up to three years after the end of the trial for future analyses of biomarkers of scientific interest.

10.0 ADVERSE EVENTS

10.1 Definitions

10.1.1 AEs

An AE is defined as any untoward medical occurrence in a clinical investigation subject administered a drug; it does not necessarily have to have a causal relationship with this treatment.

An AE can therefore be any unfavorable and unintended sign (eg, a clinically significant abnormal laboratory finding), symptom, or disease temporally associated with the use of a drug whether or not it is considered related to the drug.

10.1.2 Additional Points to Consider for AEs

An untoward finding generally may:

- Indicate a new diagnosis or unexpected worsening of a pre-existing condition. (Intermittent events for pre existing conditions underlying disease should not be considered AEs.)
- Necessitate therapeutic intervention.
- Require an invasive diagnostic procedure.
- Require discontinuation or a change in dose of study medication or a concomitant medication.
- Be considered unfavorable by the investigator for any reason.
- AEs caused by a study procedure (eg, a bruise after blood draw) should be recorded as a AE.

Diagnoses vs signs and symptoms:

- Each event should be recorded to represent a single diagnosis. Accompanying signs (including abnormal laboratory values or ECG findings) or symptoms should NOT be recorded as additional AEs. If a diagnosis is unknown, sign(s) or symptom(s) should be recorded appropriately as an AE(s).

Laboratory values and ECG findings:

- Changes in laboratory values or ECG parameters are only considered to be AEs if they are judged to be clinically significant (ie, if some action or intervention is required or if the investigator judges the change to be beyond the range of normal physiologic fluctuation). A laboratory re-test and/or continued monitoring of an abnormal value are not considered an intervention. In addition, repeated or additional noninvasive testing for verification, evaluation or monitoring of an abnormality is not considered an intervention.
- If abnormal laboratory values or ECG findings are the result of pathology for which there is an overall diagnosis (eg, increased creatinine in renal failure), the diagnosis only should be reported appropriately as an AE.

Pre-existing conditions:

- Pre-existing conditions (present at the time of signing of informed consent) are considered concurrent medical conditions and should NOT be recorded as AEs. Baseline evaluations (eg, laboratory tests, ECG, X-rays etc.) should NOT be recorded as AEs unless related to study procedures. However, if the subject experiences a worsening or complication of such a concurrent condition, the worsening or complication should be recorded appropriately as an AE (worsening or complication occurs before start of study medication) or an AE (worsening or complication occurs after start of study medication). Investigators should ensure that the event term recorded captures the change in the condition (eg, "worsening of…").
- If a subject has a pre-existing episodic condition (eg, asthma, epilepsy) any occurrence of an episode should only be captured as an AE if the episodes become more frequent, serious or severe in nature, that is, investigators should ensure that the AE term recorded captures the change in the condition from Baseline (eg "worsening of…").
- If a subject has a degenerative concurrent condition (eg, cataracts, rheumatoid arthritis), worsening of the condition should only be captured as a AE if occurring to a greater extent to that which would be expected. Again, investigators should ensure that the AE term recorded captures the change in the condition (eg, "worsening of…").

Worsening of AEs:

- If the subject experiences a worsening or complication of an AE after starting administration of the study medication, the worsening or complication should be recorded appropriately as an AE. Investigators should ensure that the AE term recorded captures the change in the condition (eg, "worsening of…").
- If the subject experiences a worsening or complication of an AE after any change in study medication, the worsening or complication should be recorded as a new AE. Investigators should ensure that the AE term recorded captures the change in the condition (eg, "worsening of…").

Changes in severity of AEs:

- If the subject experiences changes in severity of an AE, the event should be captured once with the maximum severity recorded.

Preplanned surgeries or procedures:

- Preplanned procedures (surgeries or therapies) that were scheduled prior to signing of informed consent are not considered AEs. However, if a preplanned procedure is performed early (eg, as an emergency) due to a worsening of the pre-existing condition, the worsening of the condition should be captured appropriately as an AE. Complications resulting from any planned surgery should be reported as AEs.

Elective surgeries or procedures:

- Elective procedures performed where there is no change in the subject's medical condition should not be recorded as AEs, but should be documented in the subject's source documents. Complications resulting from an elective surgery should be reported as AEs.

Insufficient clinical response (lack of efficacy):

- Insufficient clinical response, efficacy, or pharmacologic action, should NOT be recorded as an AE. The investigator must make the distinction between exacerbation of pre-existing illness and lack of therapeutic efficacy.

Overdose:

- Cases of overdose with any medication without manifested side effects are NOT considered AEs.

10.1.3 SAEs

An SAE is defined as any untoward medical occurrence that at any dose:

1. Results in DEATH.
2. Is LIFE THREATENING.
    - The term "life threatening" refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event that hypothetically might have caused death if it were more severe.
3. Requires inpatient HOSPITALIZATION or prolongation of existing hospitalization.
4. Results in persistent or significant DISABILITY/INCAPACITY.
5. Leads to a CONGENITAL ANOMALY/BIRTH DEFECT.
6. Is an IMPORTANT MEDICAL EVENT that satisfies any of the following:

- May require intervention to prevent items 1 through 5 above.
- May expose the subject to danger, even though the event is not immediately life threatening or fatal or does not result in hospitalization.
- Includes any event or synonym described in the Takeda Medically Significant AE List (Table 10.a).

Table 10.a Takeda Medically Significant AE List

Term	
Acute respiratory failure/acute respiratory distress syndrome	Hepatic necrosis
Torsade de pointes / ventricular fibrillation / ventricular tachycardia	Acute liver failure
	Anaphylactic shock
Malignant hypertension	Acute renal failure
Convulsive seizure	Pulmonary hypertension
Agranulocytosis	Pulmonary fibrosis
Aplastic anemia	Confirmed or suspected endotoxin shock
Toxic epidermal necrolysis/Stevens-Johnson syndrome	Confirmed or suspected transmission of infectious agent by a medicinal product
	Neuroleptic malignant syndrome / malignant hyperthermia
	Spontaneous abortion / stillbirth and fetal death

AEs that fulfill 1 or more of the serious criteria above are also to be considered SAEs and should be reported and followed up in the same manner (see Sections 10.2.2 and 10.3).

10.1.4 Severity of AEs

The different categories of intensity (severity) are characterized as follows:

Mild:	Transient symptoms, no interference with the Subject's daily activities.
Moderate:	Marked symptoms, moderate interference with the Subject's daily activities
Severe:	Considerable interference with the Subject's daily activities..

10.1.5 Causality of AEs

The relationship of each AE to study medication(s) will be assessed using the following categories:

Related:	An AE that follows a reasonable temporal sequence from administration of a drug (including the course after withdrawal of the drug), or for which possible involvement of the drug can be argued, although factors other than the drug, such as underlying diseases, complications, concomitant drugs and concurrent treatments, may also be responsible.
Not Related:	An AE that does not follow a reasonable temporal sequence from administration of a drug and/or that can reasonably be explained by other factors, such as underlying diseases, complications, concomitant drugs and concurrent treatments.

10.1.6 Relationship to Study Procedures

Relationship (causality) to study procedures should be determined for all AEs.

The relationship should be assessed as Related if the investigator considers that there is reasonable possibility that an event is due to a study procedure. Otherwise, the relationship should be assessed as Not Related.

10.1.7 Start Date

The start date of the AE is the date that the first signs/symptoms were noted by the subject and/or physician.

10.1.8 Stop Date

The stop date of the AE is the date at which the subject recovered, the event resolved but with sequelae or the subject died.

10.1.9 Action Concerning Study Medication

- Drug withdrawn – a study medication is stopped due to the particular AE.
- Dose not changed – the particular AE did not require stopping a study medication.
- Unknown – only to be used if it has not been possible to determine what action has been taken.
- Not Applicable – a study medication was stopped for a reason other than the particular AE eg, the study has been terminated, the subject died, dosing with study medication was already stopped before the onset of the AE.
- Dose Interrupted – the dose was interrupted due to the particular AE.

10.1.10 Outcome

- Recovered/Resolved – Subject returned to first assessment status with respect to the AE.
- Recovering/Resolving – the intensity is lowered by one or more stages: the diagnosis or signs/symptoms has almost disappeared; the abnormal laboratory value improved, but has not returned to the normal range or to baseline; the subject died from a cause other than the particular AE with the condition remaining “recovering/resolving”.
- Not recovered/not resolved – there is no change in the diagnosis, signs or symptoms; the intensity of the diagnosis, signs/ symptoms or laboratory value on the last day of the observed study period has got worse than when it started; is an irreversible congenital anomaly; the subject died from another cause with the particular AE state remaining “Not recovered/not resolved”.
- Resolved with sequelae – the subject recovered from an acute AE but was left with permanent/significant impairment (eg, recovered from a cardiovascular accident but with some persisting paresis).
- Fatal – the AEs which are considered as the cause of death.

- Unknown – the course of the AE cannot be followed up due to hospital change or residence change at the end of the subject's participation in the study.

10.2 Procedures

10.2.1 Collection and Reporting of AEs

10.2.1.1 AE Collection Period

Collection of AEs will commence from the time that the subject signs the informed consent to participate in the study continue until V7.

10.2.1.2 AE Reporting

At each study visit, the investigator will assess whether any subjective AEs have occurred. A neutral question, such as "How have you been feeling since your last visit?" may be asked. Subjects may report AEs occurring at any other time during the study. Subjects experiencing a serious AE must be monitored until the symptoms subside and any clinically relevant changes in laboratory values have returned to baseline or there is a satisfactory explanation for the change.

All subjects experiencing AEs, whether considered associated with the use of the study medication or not, must be monitored until the symptoms subside and any clinically relevant changes in laboratory values have returned to baseline or until there is a satisfactory explanation for the changes observed. All AEs will be documented in the AE page of the CRF, whether or not the investigator concludes that the event is related to the drug treatment. The following information will be documented for each event:

1. Event term.
2. Start and stop date.
3. Severity.
4. Investigator's opinion of the causal relationship between the event and administration of study medication(s) (related or not related).
5. Investigator's opinion of the causal relationship to study procedure(s), including the details of the suspected procedure.
6. Action concerning study medication.
7. Outcome of event.
8. Seriousness.

Through this follow-up if it is determined that an AE not previously reported has been identified, normal reporting requirements should be applied.

10.2.2 Collection and Reporting of SAEs

When an SAE occurs through the AE collection period it should be reported according to the following procedure:

A Takeda SAE form must be completed, in English, and signed by the investigator immediately or within 24 hours of first onset or notification of the event. The information should be completed as fully as possible but contain, at a minimum:

- A short description of the event and the reason why the event is categorized as serious.
- Subject identification number.
- Investigator's name.
- Name of the study medication(s)
- Causality assessment.

The SAE form should be transmitted within 24 hours to the attention of the contact listed in Section 1.1.

Any SAE spontaneously reported to the investigator following the routine AE collection period should be reported to the sponsor if considered related to study participation.

10.3 Follow-up of SAEs

If information not available at the time of the first report becomes available at a later date, the investigator should complete a follow-up SAE form or provide other written documentation and fax it immediately within 24 hours of receipt. Copies of any relevant data from the hospital notes (eg, ECGs, laboratory tests, discharge summary, postmortem results) should be sent to the addressee, if requested.

All SAEs should be followed up until resolution or permanent outcome of the event. The timelines and procedure for follow-up reports are the same as those for the initial report.

10.3.1 Safety Reporting to Investigators, IRBs or IECs, and Regulatory Authorities

The sponsor will be responsible for reporting all suspected unexpected serious adverse reactions (SUSARs) and any other applicable SAEs to regulatory authorities, including the European Medicines Agency (EMA), investigators and IRBs or IECs, as applicable, in accordance with national regulations in the countries where the study is conducted. Relative to the first awareness of the event by/or further provision to the sponsor or sponsor's designee, SUSARs will be submitted to the regulatory authorities as expedited report within 7 days for fatal and life-threatening events and 15 days for other serious events, unless otherwise required by national regulations. The sponsor will also prepare an expedited report for other safety issues where these might materially alter the current benefit-risk assessment of an investigational medicinal product or that would be sufficient to consider changes in the investigational medicinal products

administration or in the overall conduct of the trial. The investigational site also will forward a copy of all expedited reports to his or her IRB or IEC in accordance with national regulations.

11.0 STUDY-SPECIFIC COMMITTEES

11.1 Steering Committee

Scientific advice on the trial design, procedures and assessments has been obtained from a steering committee composed by the following scientific experts:

- , London Chest Hospital, Barts and the London Trust and Medical School, .
- Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari Università degli Studi di Padova, Unità Operativa di Pneumologia,
- , Pneumologisches Forschungsinstitut an der LungenClinic Grosshansdorf GmbH, .

The Steering Committee provides scientific advice as and when required throughout the study duration. As of , discontinuted his participation in this Steering Committee due to a potential conflict of interest in his new position with another company.

12.0 DATA HANDLING AND RECORDKEEPING

The full details of procedures for data handling will be documented in the Data Management Plan. AEs, medical history, and concurrent conditions will be coded using the Medical Dictionary for Regulatory Activities (MedDRA [SOC, HLGT, HLT, LL, PT, and their corresponding descriptive terms]). Drugs will be coded using the World Health Organization (WHO) Drug Dictionary.

12.1 CRFs

Completed CRFs are required for each subject who signs an informed consent.

The sponsor or its designee will supply investigative sites with paper CRFs. These forms are used to transmit the information collected in the performance of this study to the sponsor and regulatory authorities. CRFs must be completed in English. All paper CRFs must be filled out legibly in black or blue ballpoint ink (use of black ink is preferred).

After completion of the entry process, computer logic checks will be run to identify items, such as inconsistent dates, missing data, and questionable values. Queries may be issued by Takeda personnel (or designees) and will be answered by the site.

Corrections are to be made by making a single-line strikeout of the incorrect information and writing in the revisions. All corrections must be initialed and dated. Reasons for significant corrections should additionally be included.

The principal investigator must review the CRFs for completeness and accuracy and must sign and date the appropriate CRFs as indicated. Furthermore, the investigator must retain full responsibility for the accuracy and authenticity of all data entered on the CRFs.

CRFs will be reviewed for completeness and acceptability at the study site during periodic visits by study monitors. The sponsor or its designee will be permitted to review the subject's medical and hospital records pertinent to the study to ensure accuracy of the CRFs. The completed CRFs are the sole property of the sponsor and should not be made available in any form to third parties, except for authorized representatives of appropriate governmental health or regulatory authorities, without written permission of the sponsor.

12.2 Record Retention

The investigator agrees to keep the records stipulated in Section 12.1 and those documents that include (but are not limited to) the study-specific documents, the identification log of all participating subjects, medical records, temporary media such as thermal sensitive paper, source worksheets, all original signed and dated informed consent forms, subject authorization forms regarding the use of personal health information (if separate from the informed consent forms), copies of all paper CRFs and query responses, including the audit trail, and detailed records of drug disposition to enable evaluations or audits from regulatory authorities, the sponsor or its designees. Any source documentation printed on degradable thermal sensitive paper should be photocopied by the site and filed with the original in the subject's chart to ensure long term legibility. Furthermore, International Conference on Harmonisation (ICH) E6 Section 4.9.5

requires the investigator to retain essential documents specified in ICH E6 (Section 8) until at least 2 years after the last approval of a marketing application for a specified drug indication being investigated or, if an application is not approved, until at least 2 years after the investigation is discontinued and regulatory authorities are notified. In addition, ICH E6 Section 4.9.5 states that the study records should be retained until an amount of time specified by applicable regulatory requirements or for a time specified in the Clinical Study Site Agreement between the investigator and sponsor.

Refer to the Clinical Study Site Agreement for the sponsor's requirements on record retention. The investigator should contact and receive written approval from the sponsor before disposing of any such documents.

13.0 STATISTICAL METHODS

13.1 Statistical and Analytical Plans

A statistical analysis plan (SAP) will be prepared and finalized prior to unblinding of subject's treatment assignment. This document will provide further details regarding the definition of analysis variables and analysis methodology to address all study objectives. A data review, blinded for the main period, will be conducted prior to database lock Data handling issues and exploratory analyses may be changed due to unforeseen reasons after the blinded data review. All later deviations and/or alterations will be documented and summarised in the Clinical Trial Report.

Statistical analysis will be performed by the Sponsor's representative. The final statistical analysis must be approved by the Sponsor.

13.1.1 Analysis Sets

The following refers only to the evaluation of clinical data.

Analyses will be based on the following sets: total set, safety set, full analysis set, valid cases set. The definitions of the analysis sets are in consistency with those given in the ICH E9 Guideline (1998).

13.1.1.1 Total Set

The total set consists of all Subjects enrolled, including Subjects withdrawn prior to randomization (defined as non-eligible Subjects) and those randomized Subjects, who never took any dose of the trial treatment after randomization (defined as randomized but not treated Subjects).

13.1.1.2 Safety Set

The safety set (SAF) is based on all randomized Subjects who took at least one dose of the trial treatment after randomization. Safety analyses will be based on the SAF. For the safety analyses Subjects will be assigned to the treatment they actually received ('as treated' analysis). In addition, analyses for baseline AEs will be performed on the total set.

13.1.1.3 Full Analysis Set

The full analysis set (FAS) includes all randomized Subjects who took at least one dose of the trial treatment after randomization. In case Subjects were randomized and treated more than once, only the first randomization (and treatment) of the Subject will be included in the FAS.

The Intention-to-treat (ITT) analysis will be based on the FAS. Subjects will be assigned to the treatment group based on the treatment to which they were randomized ('as randomized' analysis).

The ITT analysis will be the primary analysis for this trial and will be performed for all primary and secondary endpoints.

13.1.1.4 Valid Cases Set

The valid cases set (VCS) consists of all Subjects of the FAS without any major protocol violations. Subjects terminating prematurely will be included in the VCS provided that there is no major protocol violation.

Subjects with major protocol violations considering the inclusion criteria, randomization criteria, and criteria affecting the read-out parameters of the trial will be identified during reviewof the blinded data. Violation of the criteria within ethical considerations in terms of general health are considered minor deviations in terms of impact on interpretability of the data, but it will be assessed during review of the blinded data if there is an impact for single cases. Deviations occurring during the following procedures will not be considered major protocol violations for the primary objective of the trial: Telephone contacts after early withdrawal and follow-up visit after regular trial end.

The Per-Protocol (PP) analysis is based on the VCS. In analogy to the ITT analysis it will be performed on an 'as randomized' basis.

The PP analysis will be used to assess the robustness of the results and will be performed for the primary and key secondary endpoints (see Section 13.1.3.2) only.

13.1.2 Analysis of Demographics and Other Baseline Characteristics

Demographic and baseline characteristics will be summarized by each treatment group and overall. Summary statistics (N, mean, SD, median, minimum, and maximum) will be generated for continuous variables (eg, age and weight) and the number and percentage of subjects within each category will be presented for categorical variables (eg, sex, and race). Individual subject demographic and baseline characteristics data will be listed.

13.1.3 Efficacy Analysis

For the primary analysis all data until a Subject discontinued (prematurely or as scheduled) will be included. All data that was captured after a Subject discontinued the trial or at the Follow-up visit (V7) will be included in descriptive analyses. These additional analyses will be considered as ITT analyses.

The analysis of primary and secondary endpoints will be based on the FAS, the subset of the FAS without exacerbation during double-blind treatment, and the VCS (see below for details).

13.1.3.1 Primary analysis of primary and key secondary endpoints

The primary analysis will be performed in the FAS.

The **primary variable** relates to pulmonary inflammation expressed as inflammatory CD8+ cell counts per mm^2 in sub-mucosal bronchial biopsy tissue specimen measured before and after the double-blind treatment period (i.e. at V2 and at V6). CD68+ cell counts per mm^2 in sub-mucosal bronchial biopsy tissue specimen at V2 and at V6 is the **key secondary variable**.

Roflumilast/placebo comparisons with respect to CD8+ and CD68+ will be done via a multiple test procedure such that the family-wise error rate of 5% is controlled in the strong sense. The two null hypotheses are: equal CD8+ counts/mm^2 and equal CD68+ counts/mm^2 on Roflumilast and placebo. These null hypotheses will be ordered, so that the CD8+ comparison comes first and the CD68+ comparison comes second. If the comparison with respect to CD8+ is significant at the nominal level alpha = 5%, the corresponding null hypothesis will be rejected and the CD68+ comparison will be performed in a confirmatory way (otherwise confirmatory testing stops). This will again be done at nominal level alpha = 5%. If significant (after a significant result in the first comparison), the corresponding null hypothesis will be rejected. If the first comparison is not significant at the nominal 5% level, then no null hypothesis must be rejected.

The component tests of the multiple test on CD8+ and CD68+ will be based on Poisson regression models with CD8+ (CD68+) at visit V6 as dependent variable and treatment and baseline value of the respective dependent variable as covariates. A dispersion parameter and an offset (= bronchoscopy sampling area) will be taken into account. A formal description of the model and test for an inflammatory cell is this:

Let Y_{ij} be independent random variables which represent numbers of inflammatory cells in tissue area t_{ij} for treatment i (i=1: Roflumilast, i=0: placebo) and Subject j. The Y_{ij} are assumed to follow a Poisson regression model with expectation $\gamma_i = E(Y_{ij} / t_{ij})$ (not depending on Subject) and a dispersion parameter. The log-link function is assumed for the model. Log-transformation of the expectation gives the following linear function

$$\log E(Y_{ij}) = \alpha + \beta_0 \cdot X_0 + \beta_1 \cdot X_1 + \log(t_{ij}),$$

where α is an intercept, X_0 is the baseline value (=cell count per mm^2 at baseline V2), X_1 the treatment indicator (1=Roflumilast, 0=placebo) and β_0 and β_1 are regression coefficients.

The null hypothesis is

$$H_0 : \beta_1 = 0.$$

This means that the number of cells per tissue area on Roflumilast and placebo are the same.

H_0 will be tested using the likelihood ratio statistic.

13.1.3.2 Supportive analysis of primary and key secondary endpoints

The following analyses will be performed in the FAS, FAS without exacerbation during double-blind treatment and VCS.

- Descriptive: absolute and change variables (change from V2 to V6)
- ANCOVA on change variables (from V2 to V6) with covariates as in the primary analysis plus stratification factor as covariate. Point estimates of adjusted treatment differences will be presented with confidence intervals.

- Poisson regression with covariates as in the primary analysis without confirmatory testing (i.e. p-values associated with treatment effect are interpreted along exploratory lines). Point estimates of adjusted treatment ratios will be presented with confidence intervals.
- Descriptive: absolute and change variables (change from V2 to V6) in subgroups formed by COPD stage, smoking status, concomitant LABA use and former ICS use (4 subgroup analyses).
- Poisson regressions with covariates as in the primary analysis plus covariates indicating COPD stage, smoking status, concomitant LABA use and former ICS use (4 different models) without confirmatory testing. Point estimates of adjusted treatment ratios with confidence intervals will be presented

13.1.3.3 Analysis of secondary endpoints

These analyses will be performed in the FAS (but the key secondary variable CD68+ cell count will be analysed as described above). They will be descriptive by treatment and visit (all visits from V1 or V2 to V5 or V6 with data available, pulmonary function data include V0), by ANCOVA on absolute change from baseline (V1 or V2 depending on parameter) to last available measurement during double-blind treatment (for Subjects with complete data V5 or V6 depending on parameter) for continuous variables (covariates: baseline value, treatment, stratification factor) or Poisson regression with dispersion parameter for count data at visit with last measurement on double-blind treatment (completers: V5 or V6; covariates: baseline count, treatment, stratification factor). In Poisson regression models the area investigated will be included as an offset variable in the model.

- **Cell count in biopsied material (sub-mucosa) [cells/mm^2]:**
  1) CD68+ descriptive, Poisson regression (key secondary endpoint): see Sections 13.1.3.1. and 13.1.3.2
  2) Neutrophils: descriptive, Poisson regression
  3) CD4+: descriptive, Poisson regression
  4) CD45+: descriptive, Poisson regression
- **Cell count in biopsied material (bronchial epithelium) [cells/mm^2]:**
  1) CD8+: descriptive, Poisson regression
  2) CD68+: descriptive, Poisson regression
- **Total and differential cell counts in induced sputum absolute [cells/mL]:**
  1) Neutrophils: descriptive, ANCOVA
  2) Macrophages: descriptive, ANCOVA
  3) Eosinophils: descriptive, ANCOVA

4) Lymphocytes: descriptive, ANCOVA

- **percentage [%]:**
  1) Neutrophils: descriptive, ANCOVA
  2) Macrophages: descriptive, ANCOVA
  3) Eosinophils: descriptive, ANCOVA
  4) Lymphocytes : descriptive, ANCOVA
- **Concentration of inflammatory biomarkers in sputum:**
  1) Inflammatory mediators: descriptive, ANCOVA
- **Concentration of inflammatory biomarkers in blood serum:**
  2) Inflammatory mediators: descriptive, ANCOVA
- **Metabolomics**

  The data from the measurement of a panel of different metabolites in blood serum will be analysed in exploratory terms. The metabolomics analyses will be described in a separate statistical analysis plan. Results will go into a separate report. These analyses will not be part of the main SAP or main CSR.
- **Pulmonary function changes in the course of the trial:**

  Change from randomization (V2) over 16 weeks of treatment for:
  1) FEV_1 [L], FVC [L], FEV_1/FVC [%]: descriptive

13.1.4 Other Analyses

Health economic evaluations are not planned.

The analysis for the following exploratory objectives will be described in a separate statistical analysis plan and the results will go into a separate report. These analyses will not be part of the main SAP or main CSR.

- **Microbiome:** The interaction between changes in the lung microbiota, airway inflammation and mucin production resulting from roflumilast therapy (Appendix I)
- **Transcriptome**: To study the molecular anti-inflammatory treatment effects of Roflumilast in study RO-2455-402-RD (Appendix J)
- **Airway Reflux:** An exploratory research approach to study the changes of the degree of airway reflux (Appendix K)

13.1.5 Safety Analysis

The safety analyses will be performed in the SAF.

13.1.5.1 Adverse Events

Incidences of AEs will be summarized by MedDRA preferred term (PT) and system organ class (SOC). Such summaries will be provided for all baseline AEs, all treatment-emergent AEs, treatment-emergent AEs leading to death, treatment-emergent AEs leading to withdrawal, treatment-emergent non-serious AEs, treatment-emergent serious AEs, treatment-emergent serious AEs leading to withdrawal, treatment-emergent AEs judged to be related (Investigator) to the trial treatment or to trial procedures, treatment-emergent serious AEs judged to be related (Investigator, Investigator and/or Sponsor) to the trial treatment, and treatment-emergent AEs judged to be related to the trial treatment and leading to withdrawal. In addition treatment-emergent AEs (SOC and PTs) will be summarised also by intensity, association to the trial treatment, outcome, time to onset and duration. Preferred terms by descending frequencies will be provided for all treatment-emergent AEs, treatment-emergent AEs judged to be related to the trial treatment, treatment-emergent serious AEs, and treatment-emergent AEs leading to withdrawal.

Further details regarding the analysis of AEs will be given in the SAP.

13.1.5.2 Clinical laboratory evaluations, ECGs, vital signs

Clinical laboratory data will be presented on an individual basis and will be marked according to the normal ranges provided by the selected central laboratory. Descriptive summary statistics, including changes from randomization (V2), will be presented for each variable. In addition, the number of values outside the normal ranges and outside extended ranges will be presented.

The data from ECG and vital signs will be presented in a descriptive manner as well.

13.1.5.3 Body weight and BMI

Weight change [kg] and BMI change [kg/m^2] will be analysed using an ANCOVA model including LOCF. The dependent variable will be the change from randomization (V2) to the last scheduled post-randomization visit up to visit V6. The following factors and covariates (all fixed) will be included in the model: treatment and baseline value.

In addition, pre-treatment with LABA (yes/no) will be included in the model as this is used as stratification factor in the randomization (CPMP/EWP/2863/99) [25].

Descriptive summary statistics (N, mean, SD, median, 68% range, minimum and maximum) will be provided for all scheduled visits (absolute values as well as changes from baseline) by treatment. A Subject data listing by treatment and visit will be provided showing body weight [kg] and BMI [kg/m^2] (absolute values as well as changes from baseline).

Further details regarding body weight and BMI will be given in the SAP.

13.2 Interim Analysis and Criteria for Early Termination

No interim analysis is planned.

13.3 Determination of Sample Size

Based on the extensive literature search/review and experimental data provided by the Steering Committee (SC) members the team concludes that significant differences exist between reports published by various groups, which may be attributable to different patient populations, bronchoscopy/tissue harvesting/processing methods and statistical methods employed (see attached table below).

Additionally based on SC members' opinion a universally recognized level of clinical relevance regarding the first primary variable (sub-mucosa CD8+ cells) has not yet been agreed upon within the scientific community of chest physicians and pathologists. But it has been indicated by SC members that a 30% improvement on Roflumilast over placebo may be of clinical relevance.

Following the analysis of literature data, SC members' opinion and for feasibility reasons the total number of enrolled Subjects has been capped at 150 in total.

The literature gives some guidance on expected drop-out rates in such a trial:

Citation	Patients screened	Randomized	Discontinued trial	Completed trial (had 2 bronchoscopies)
Bourbeau et al. 2007 [26]	62	60	9	51
Lapperre et al. 2009 [11]		114		90
Gamble et al. 2003 [21]	77	59	6	53
Hattotuwa et al. 2002(b) [27]		37		31
Barnes et al. 2006 [14]	162	140		114

The table above indicates that drop-out rates (expressed as a percentage of enrolled Subjects) may be as high as 30%. To have a conservative estimate a 30% drop-out rate is assumed in this trial. Thus, the trial may end up with 105 Subjects having two bronchoscopies.

The subsequent table below contains some example calculations for sample size. They are for the primary variable (subepithelial CD8+ cell count/mm^2). Analysis by a Poisson regression model with dispersion parameter is assumed. Sample size was calculated according to formula (4.3) of Yee, 1998 [28]. Further assumptions were the following: 1:1 randomization, two-sided alpha = 0.05, power = 0.90, 0.85, 0.80, event rate on Roflumilast = 200 cells/mm^2, event rate on placebo 285 cells/mm^2. Such a reduction of event rates would correspond to an improvement of ca. 30% on Roflumilast (placebo event rate corresponds to 100%). Given the uncertainty in the assumptions around the mean area examined and the counts per mm^2, Takeda may conduct a blinded pooled assessment of these quantities and revise the sample size to ensure the study remains adequately powered.

Total number of evaluable Subjects needed by mean tissue area and variability of measurements as expressed by dispersion parameter*).

Power	**Mean area examined**	**Dispersion parameter**			
		20	**25**	**60**	**100**
90%	0.3 mm^2	96	120	286	476
90%	0.4 mm^2	72	90	214	358
85%	0.3 mm^2	82	102	244	408
85%	0.4 mm^2	62	78	184	306
80%	0.3 mm^2	72	90	214	356
80%	0.4 mm^2	54	68	160	268

*) Variance = dispersion parameter x Poisson expectation.

Thus, with a dispersion of 25 and a tissue area of at least 0.3 mm^2 and further assumptions as above, the trial would have a high power (ca. 90%) to detect treatment differences.

14.0 QUALITY CONTROL AND QUALITY ASSURANCE

14.1 Study-Site Monitoring Visits

Monitoring visits to the study site will be made periodically during the study to ensure that all aspects of the protocol are followed. Source documents will be reviewed for verification of data recorded on the CRFs. Source documents are defined as original documents, data, and records. The investigator and institution guarantee access to source documents by the sponsor or its designee (CRO) and by the IRB or IEC.

All aspects of the study and its documentation will be subject to review by the sponsor or designee (as long as blinding is not jeopardized), including but not limited to the Investigator's Binder, study medication, subject medical records, informed consent documentation, documentation of subject authorization to use personal health information (if separate from the informed consent forms), and review of CRFs and associated source documents. It is important that the investigator and other study personnel are available during the monitoring visits and that sufficient time is devoted to the process.

14.2 Protocol Deviations

The investigator should not deviate from the protocol, except where necessary to eliminate an immediate hazard to study subjects. Should other unexpected circumstances arise that will require deviation from protocol-specified procedures, the investigator should consult with the sponsor or designee (and IRB or IEC, as required) to determine the appropriate course of action. There will be no exemptions (a prospectively approved deviation) from the inclusion or exclusion criteria.

The site should document all protocol deviations in the subject's source documents. In the event of a significant deviation, the site should notify the sponsor or its designee (and IRB or EC, as required). Significant deviations include, but are not limited to, those that involve fraud or misconduct, increase the health risk to the subject, or confound interpretation of primary study assessment. A Protocol Deviation Form should be completed by the site and signed by the sponsor or designee for any significant deviation from the protocol.

14.3 Quality Assurance Audits and Regulatory Agency Inspections

The study site also may be subject to quality assurance audits by the sponsor or designees. In this circumstance, the sponsor-designated auditor will contact the site in advance to arrange an auditing visit. The auditor may ask to visit the facilities where laboratory samples are collected, where the medication is stored and prepared, and any other facility used during the study. In addition, there is the possibility that this study may be inspected by regulatory agencies, including those of foreign governments (eg, the FDA, the United Kingdom Medicines and Healthcare products Regulatory Agency, the Pharmaceuticals and Medical Devices Agency of Japan). If the study site is contacted for an inspection by a regulatory body, the sponsor should be notified immediately. The investigator and institution guarantee access for quality assurance auditors to all study documents as described in Section 14.1.

15.0 ETHICAL ASPECTS OF THE STUDY

This study will be conducted with the highest respect for the individual participants (i.e, subjects) according to the protocol, the ethical principles that have their origin in the Declaration of Helsinki, and the ICH Harmonised Tripartite Guideline for GCP. Each investigator will conduct the study according to applicable local or regional regulatory requirements and align his or her conduct in accordance with the "Responsibilities of the Investigator" that are listed in Appendix A Schedule of Study Procedures . The principles of Helsinki are addressed through the protocol and through appendices containing requirements for informed consent and investigator responsibilities

This trial was designed and will be performed according to the European Medicines Agency (EMA) recommendations CPMP/EWP/562/98: 'Points to consider on clinical investigation of medicinal products in the chronic treatment of patients with chronic obstructive pulmonary disease (COPD)' [29] and the EMA concept paper (CHMP/EWP/8197/2009) on the need for revisions of the aforementioned points to consider [30].

15.1 IRB and/or IEC Approval

IRBs and IECs must be constituted according to the applicable local requirements of each participating region. The sponsor or designee will require documentation noting all names and titles of members who make up the respective IRB or IEC. If any member of the IRB or IEC has direct participation in this study, written notification regarding his or her abstinence from voting must also be obtained.

The sponsor or designee will supply relevant documents for submission to the respective IRB or IEC for the protocol's review and approval. This protocol, the Investigator's Brochure, a copy of the informed consent form, and, if applicable, subject recruitment materials and/or advertisements and other documents required by all applicable laws and regulations, must be submitted to a central or local IRB or IEC for approval. The IRB's or IEC's written approval of the protocol and subject informed consent must be obtained and submitted to the sponsor or designee before commencement of the study (ie, before shipment of the sponsor-supplied drug or study specific screening activity). The IRB or IEC approval must refer to the study by exact protocol title, number, and version date; identify versions of other documents (eg, informed consent form) reviewed; and state the approval date. The sponsor will notify site once the sponsor has confirmed the adequacy of site regulatory documentation and, when applicable, the sponsor has received permission from competent authority to begin the trial. Until the site receives notification no protocol activities, including screening may occur.

Sites must adhere to all requirements stipulated by their respective IRB or IEC. This may include notification to the IRB or IEC regarding protocol amendments, updates to the informed consent form, recruitment materials intended for viewing by subjects, local safety reporting requirements, reports and updates regarding the ongoing review of the study at intervals specified by the respective IRB or IEC, and submission of the investigator's final status report to IRB or IEC. All

IRB and IEC approvals and relevant documentation for these items must be provided to the sponsor or its designee.

Subject incentives should not exert undue influence for participation. Payments to subjects must be approved by the IRB or IEC and sponsor.

15.2 Subject Information, Informed Consent, and Subject Authorization

Written consent documents will embody the elements of informed consent as described in the Declaration of Helsinki and the ICH Guidelines for GCP and will be in accordance with all applicable laws and regulations. The informed consent form, subject authorization form (if applicable), and subject information sheet (if applicable) describe the planned and permitted uses, transfers, and disclosures of the subject's personal and personal health information for purposes of conducting the study. The informed consent form and the subject information sheet (if applicable) further explain the nature of the study, its objectives, and potential risks and benefits, as well as the date informed consent is given. The informed consent form will detail the requirements of the participant and the fact that he or she is free to withdraw at any time without giving a reason and without prejudice to his or her further medical care.

The investigator is responsible for the preparation, content, and IRB or IEC approval of the informed consent form and if applicable, the subject authorization form. The informed consent form, subject authorization form (if applicable), and subject information sheet (if applicable) must be approved by both the IRB or IEC and the sponsor prior to use.

The informed consent form, subject authorization form (if applicable), and subject information sheet (if applicable) must be written in a language fully comprehensible to the prospective subject. It is the responsibility of the investigator to explain the detailed elements of the informed consent form, subject authorization form (if applicable), and subject information sheet (if applicable) to the subject. Information should be given in both oral and written form whenever possible and in the manner deemed appropriate by the IRB or IEC. In the event the subject is not capable of rendering adequate written informed consent, then the subject's legally acceptable representative may provide such consent for the subject in accordance with applicable laws and regulations.

The subject, or the subject's legally acceptable representative, must be given ample opportunity to: (1) inquire about details of the study and (2) decide whether or not to participate in the study. If the subject, or the subject's legally acceptable representative, determines he or she will participate in the study, then the informed consent form and subject authorization form (if applicable) must be signed and dated by the subject, or the subject's legally acceptable representative, at the time of consent and prior to the subject entering into the study. The subject or the subject's legally acceptable representative should be instructed to sign using their legal names, not nicknames, using blue or black ballpoint ink. The investigator must also sign and date the informed consent form and subject authorization (if applicable) at the time of consent and prior to subject entering into the study; however, the sponsor may allow a designee of the investigator to sign to the extent permitted by applicable law.

Once signed, the original informed consent form, subject authorization form (if applicable), and subject information sheet (if applicable) will be stored in the investigator's site file. The investigator must document the date the subject signs the informed consent in the subject's medical record. Copies of the signed informed consent form, the signed subject authorization form (if applicable), and subject information sheet (if applicable) shall be given to the subject.

All revised informed consent forms must be reviewed and signed by relevant subjects or the relevant subject's legally acceptable representative in the same manner as the original informed consent. The date the revised consent was obtained should be recorded in the subject's medical record, and the subject should receive a copy of the revised informed consent form.

15.3 Subject Confidentiality

The sponsor and designees affirm and uphold the principle of the subject's right to protection against invasion of privacy. Throughout this study, a subject's source data will only be linked to the sponsor's clinical study database or documentation via a unique identification number. As permitted by all applicable laws and regulations, limited subject attributes, such as sex, age, or date of birth, and subject initials may be used to verify the subject and accuracy of the subject's unique identification number.

To comply with ICH Guidelines for GCP and to verify compliance with this protocol, the sponsor requires the investigator to permit its monitor or designee's monitor, representatives from any regulatory authority (eg, FDA, Medicines and Healthcare products Regulatory Agency, Pharmaceuticals and Medical Devices Agency), the sponsor's designated auditors, and the appropriate IRBs and IECs to review the subject's original medical records (source data or documents), including, but not limited to, laboratory test result reports, ECG reports, admission and discharge summaries for hospital admissions occurring during a subject's study participation, and autopsy reports. Access to a subject's original medical records requires the specific authorization of the subject as part of the informed consent process (see Section 15.2).

Copies of any subject source documents that are provided to the sponsor must have certain personally identifiable information removed (ie, subject name, address, and other identifier fields not collected on the subject's CRF).

15.4 Publication, Disclosure, and Clinical Trial Registration Policy

15.4.1 Publication and Disclosure

The investigator is obliged to provide the sponsor with complete test results and all data derived by the investigator from the study. During and after the study, only the sponsor may make study information available to other study investigators or to regulatory agencies, except as required by law or regulation. Except as otherwise allowable in the clinical study site agreement, any public disclosure (including publicly accessible websites) related to the protocol or study results, other than study recruitment materials and/or advertisements, is the sole responsibility of the sponsor.

The sponsor may publish any data and information from the study (including data and information generated by the investigator) without the consent of the investigator. Manuscript authorship for any peer-reviewed publication will appropriately reflect contributions to the production and review of the document. All publications and presentations must be prepared in accordance with this section and the Clinical Study Site Agreement. In the event of any discrepancy between the protocol and the Clinical Study Site Agreement, the Clinical Study Site Agreement will prevail.

15.4.2 Clinical Trial Registration

In order to ensure that information on clinical trials reaches the public in a timely manner and to comply with applicable laws, regulations and guidance, Takeda will, at a minimum register interventional clinical trials it sponsors anywhere in the world on ClinicalTrials.gov or other publicly accessible websites before start of study, as defined in Takeda Policy/Standard. Takeda contact information, along with investigator's city, country, and recruiting status will be registered and available for public viewing.

For some registries, Takeda will assist callers in locating trial sites closest to their homes by providing the investigator name, address, and phone number to the callers requesting trial information. Once subjects receive investigator contact information, they may call the site requesting enrollment into the trial. The investigative sites are encouraged to handle the trial inquiries according to their established subject screening process. If the caller asks additional questions beyond the topic of trial enrollment, they should be referred to the sponsor.

Any investigator who objects to Takeda providing this information to callers must provide Takeda with a written notice requesting that their information not be listed on the registry site.

15.4.3 Clinical Trial Results Disclosure

Takeda will post the results of clinical trials on ClinicalTrials.gov or other publicly accessible websites, as required by Takeda Policy/Standard, applicable laws and/or regulations.

15.5 Insurance and Compensation for Injury

Each subject in the study must be insured in accordance with the regulations applicable to the site where the subject is participating. If a local underwriter is required, then the sponsor or sponsor's designee will obtain clinical study insurance against the risk of injury to clinical study subjects. Refer to the Clinical Study Site Agreement regarding the sponsor's policy on subject compensation and treatment for injury. If the investigator has questions regarding this policy, he or she should contact the sponsor or sponsor's designee.

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Appendix A Schedule of Study Procedures

Activities and assessments	**Run-in period**			**Double-blind treatment period**				**FU safety visit** [a]
			R				**VE**	
Visit	**V0**	**V1**	**V2**	**V3**	**V4**	**V5**	**V6**	**V7**
Weeks	**-6**	**-2**	**0**	≤2	**6**	**14**	**16**	**≤18**
Visit Window (days) [b]		-2 / +3	-3 / +5		-3 / +5	-3 / +5	-7 / +7	
Subject Information and Informed Consent	X							
Demographic data	X							
Medical History and concomitant diseases	X							
COPD history	X							
Inclusion/Exclusion criteria	X							
Withdrawal of disallowed medications	X							
Previous and concomitant medications	X	X	X	X	X	X	X	X
Substance use (smoking status)	X	X	X		X	X	X	
Physical examination	X		X				X	X
Vital signs and body measurements (including body weight and body height) [c]	X	X	X	X	X	X	X	X
Subject fasting, avoidance of strenuous exercise and smoking	X	X[d]	X	X*	X*	X*	X	X*
Standard laboratory (including blood pregnancy test) [e]	X	X[d]					X	
Urine analysis	X							
Urine pregnancy test [e]		X	X		X	X		
Clotting time and platelet count	X	X				X		
Blood sampling for assessment of inflammatory biomarkers and metabolites in blood serum		X			X	X		
Sputum induction and sputum sample processing		X			X	X		
Bronchoscopy/bronchial biopsy			X				X	
Pulse oximetry measurement			X				X	
Chest X-ray or CT scan [f] ***GERMANY ONLY:*** Check of historical chest X-ray or CT scan [f]	X							
ECG (12-lead) at rest [g]	X							
Pulmonary function test	X		X				X	

Activities and assessments	**Run-in period**			**Double-blind treatment period**				**FU safety visit** [a]
Randomization criteria check			X					
Randomization			X					
ID card dispensed	X							
Adverse event assessment (see Section 10.0)	X	X	X	X	X	X	X	X
Dispense trial treatment	X		X		X			
Trial treatment returned			X		X		X	
Tablet compliance check			X[h]		X		X	
Termination of double-blind treatment phase of the trial							X	

Notes:

- V3 and V7, which are safety visits only, are to be conducted within two weeks after bronchoscopy. The exact timing remains at the discretion of the Investigator.
- Assessments described under V6 (VE) are mandatory for randomized Subjects upon trial completion either prematurely or as scheduled.
- In selected Subjects three protected brush specimen in a middle lobe bronchus will be collected at visits V2 and V6. This is described in a separate sub-trial protocol. With regard to Subject consent and information this sub-trial is independent from the RO-2455-402-RD trial protocol (see Section 9.1.15.3).
- *For visits where subjects will not undergo blood draws or biopsies, the fasting requirement will only be mandated if clinically indicated as per investigator judgement.

Abbreviations:

R - Randomization (V2); V0 - visit 0, VE - visit end (V6), FU safety visit - follow-up safety visit (V7)

[a] Applies only to Subjects, who underwent the second bronchoscopy/bronchial biopsy at V6.

[b] Time windows for the run-in period always refer to V0, whereas those for the treatment period always refer to the randomization visit V2.

[c] Body height measurement only at visit V0.

[d] If confirmation or check of V0 laboratory values is necessary. Pregnancy test is optional.

[e] For female Subjects of childbearing potential or less than 1 year postmenopausal.

[f] If not performed within past three months. Examination results must be available.

GERMANY ONLY: Every patient should have a historical X-ray or CT scan available to clarify the exclusion criteria #18.

[g] This should be done prior to pulmonary function test.

[h] Tablet compliance calculation by the investigational site (see Section 9.2) to check for randomization criterion no. 2 (see Section 7.3).

Appendix B Responsibilities of the Investigator

Clinical research studies sponsored by the sponsor are subject to ICH GCP and all the applicable local laws and regulations.

The investigator agrees to assume the following responsibilities:

1. Conduct the study in accordance with the protocol.
2. Personally conduct or supervise the staff who will assist in the protocol.
3. Ensure that study related procedures, including study specific (non routine/non standard panel) screening assessments are NOT performed on potential subjects, prior to the receipt of written approval from relevant governing bodies/authorities.
4. Ensure that all colleagues and employees assisting in the conduct of the study are informed of these obligations.
5. Secure prior approval of the study and any changes by an appropriate IRB/IEC that conform to ICH, and local regulatory requirements.
6. Ensure that the IRB/IEC will be responsible for initial review, continuing review, and approval of the protocol. Promptly report to the IRB/IEC all changes in research activity and all anticipated risks to subjects. Make at least yearly reports on the progress of the study to the IRB/IEC, and issue a final report within 3 months of study completion.
7. Ensure that requirements for informed consent, as outlined in ICH and local regulations, are met.
8. Obtain valid informed consent from each subject who participates in the study, and document the date of consent in the subject's medical chart. Valid informed consent is the most current version approved by the IRB/IEC. Each informed consent form should contain a subject authorization section that describes the uses and disclosures of a subject's personal information (including personal health information) that will take place in connection with the study. If an informed consent form does not include such a subject authorization, then the investigator must obtain a separate subject authorization form from each subject or the subject's legally acceptable representative.
9. Prepare and maintain adequate case histories of all persons entered into the study, including CRFs, hospital records, laboratory results, etc, and maintain these data for a minimum of 2 years following notification by the sponsor that all investigations have been discontinued or that the regulatory authority has approved the marketing application. The investigator should contact and receive written approval from the sponsor before disposing of any such documents.
10. Allow possible inspection and copying by the regulatory authority of GCP-specified essential documents.
11. Maintain current records of the receipt, administration, and disposition of sponsor-supplied drugs, and return all unused sponsor-supplied drugs to the sponsor.

12. Report adverse reactions to the sponsor promptly. In the event of an SAE, notify the sponsor within 24 hours.

Appendix C Elements of the Subject Informed Consent

In seeking informed consent, the following information shall be provided to each subject:

1. A statement that the study involves research.
2. An explanation of the purposes of the research.
3. The expected duration of the subject's participation.
4. A description of the procedures to be followed, including invasive procedures.
5. The identification of any procedures that are experimental.
6. The estimated number of subjects involved in the study.
7. A description of the subject's responsibilities.
8. A description of the conduct of the study.
9. A statement describing the treatment(s) and the probability for random assignment to each treatment.
10. A description of the possible side effects of the treatment that the subject may receive.
11. A description of any reasonably foreseeable risks or discomforts to the subject and, when applicable, to an embryo, fetus, or nursing infant.
12. A description of any benefits to the subject or to others that reasonably may be expected from the research. When there is no intended clinical benefit to the subject, the subject should be made aware of this.
13. Disclosures of appropriate alternative procedures or courses of treatment, if any, that might be advantageous to the subject and their important potential risks and benefits.
14. A statement describing the extent to which confidentiality of records identifying the subject will be maintained, and a note of the possibility that regulatory agencies, auditor(s), IRB/IEC, and the monitor may inspect the records. By signing a written informed consent form, the subject or the subject's legally acceptable representative is authorizing such access.
15. For research involving more than minimal risk, an explanation as to whether any compensation and an explanation as to whether any medical treatments are available if injury occurs and, if so, what they consist of or where further information may be obtained.
16. The anticipated prorated payment(s), if any, to the subject for participating in the study.
17. The anticipated expenses, if any, to the subject for participating in the study.
18. An explanation of whom to contact for answers to pertinent questions about the research (investigator), subject's rights, and IRB/IEC and whom to contact in the event of a research-related injury to the subject.

19. A statement that participation is voluntary, that refusal to participate will involve no penalty or loss of benefits to which the subject otherwise is entitled, and that the subject may discontinue participation at any time without penalty or loss of benefits to which the subject is otherwise entitled.
20. The consequences of a subject's decision to withdraw from the research and procedures for orderly termination of participation by the subject.
21. A statement that the subject or the subject's legally acceptable representative will be informed in a timely manner if information becomes available that may be relevant to the subject's willingness to continue participation in the study.
22. A statement that results of pharmacogenomic analysis will not be disclosed to an individual, unless prevailing laws require the sponsor to do so.
23. The foreseeable circumstances or reasons under which the subject's participation in the study may be terminated.
24. A written subject authorization (either contained within the informed consent form or provided as a separate document) describing to the subject the contemplated and permissible uses and disclosures of the subject's personal information (including personal health information) for purposes of conducting the study. The subject authorization must contain the following statements regarding the uses and disclosures of the subject's personal information:

a) that personal information (including personal health information) may be processed by or transferred to other parties in other countries for clinical research and safety reporting purposes, including, without limitation, to the following: (1) Takeda, its affiliates, and licensing partners; (2) business partners assisting Takeda, its affiliates, and licensing partners; (3) regulatory agencies and other health authorities; and (4) IRBs/IECs;

b) it is possible that personal information (including personal health information) may be processed and transferred to countries that do not have data protection laws that offer subjects the same level of protection as the data protection laws within this country; however, Takeda will make every effort to keep your personal information confidential, and your name will not be disclosed outside the clinic unless required by law;

c) that personal information (including personal health information) may be added to Takeda's research databases for purposes of developing a better understanding of the safety and effectiveness of the study medication(s), studying other therapies for patients, developing a better understanding of disease, and improving the efficiency of future clinical studies;

d) that subjects agree not to restrict the use and disclosure of their personal information (including personal health information) upon withdrawal from the study to the extent that the restricted use or disclosure of such information may impact the scientific integrity of the research; and

e) that the subject's identity will remain confidential in the event that study results are published.

25. Female subjects of childbearing potential (eg, nonsterilized, premenopausal female subjects) who are sexually active must use adequate contraception (as defined in the informed consent) from Screening throughout the duration of the study. Regular pregnancy tests will be performed throughout the study for all female subjects of childbearing potential. If a subject is found to be pregnant during study, study medication will be discontinued and the investigator will offer the subject the choice to receive unblinded treatment information.
26. A statement that clinical trial information from this trial will be publicly disclosed in a publicly accessible website, such as ClinicalTrials.gov.

Appendix D Investigator Consent to Use of Personal Information

Takeda will collect and retain personal information of investigator, including his or her name, address, and other personally identifiable information. In addition, investigator's personal information may be transferred to other parties located in countries throughout the world (eg, the United Kingdom, United States, and Japan), including the following:

- Takeda, its affiliates, and licensing partners.
- Business partners assisting Takeda, its affiliates, and licensing partners.
- Regulatory agencies and other health authorities.
- IRBs and IECs.

Investigator's personal information may be retained, processed, and transferred by Takeda and these other parties for research purposes including the following:

- Assessment of the suitability of investigator for the study and/or other clinical studies.
- Management, monitoring, inspection, and audit of the study.
- Analysis, review, and verification of the study results.
- Safety reporting and pharmacovigilance relating to the study.
- Preparation and submission of regulatory filings, correspondence, and communications to regulatory agencies relating to the study.
- Preparation and submission of regulatory filings, correspondence, and communications to regulatory agencies relating to other medications used in other clinical studies that may contain the same chemical compound present in the study medication.
- Inspections and investigations by regulatory authorities relating to the study.
- Self-inspection and internal audit within Takeda, its affiliates, and licensing partners.
- Archiving and audit of study records.
- Posting investigator site contact information, study details and results on publicly accessible clinical trial registries, databases, and websites.

Investigator's personal information may be transferred to other countries that do not have data protection laws that offer the same level of protection as data protection laws in investigator's own country.

Investigator acknowledges and consents to the use of his or her personal information by Takeda and other parties for the purposes described above.

Appendix E Laboratory and Vital Sign Alert Values (Sponsor defined)

HEMATOLOGY

Hemoglobin: male < 11.5 g/dL female < 9.5 g/dL
Erythrocytes: male < 3.5 10^6/µL or > 7 10^6/µL female < 3.0 10^6/µL or > 6.5 10^6/µL
White Blood Cell Count (WBC): < 2,800/mm³ or > 16,000/mm³
Eosinophils: > 20%
Platelet Count: < 75,000/mm³ or > 700,000/mm³

CHEMISTRY

SGOT (ASAT): > 3x ULNR
SGPT (ALAT): > 3x ULNR
Gamma-GT: > 3x ULNR
Alkaline Phosphatase (AP): > 3x ULNR
CK: > 3x ULNR;
Creatinine: > 2x ULNR
Total Bilirubin: > 2x ULNR
Potassium: > 6.0 mmol/L or < 3.0 mmol/L
Sodium: > 150 mmol/L or < 130 mmol/L
Glucose: > 2 x ULNR

VITAL SIGNS

BP systolic: > 170 mmHg or < 85 mmHg
BP diastolic: > 105 mmHg
Difference systolic BP at Vx (increase or decrease) compared to pre-treatment > 40 mmHg
Resting Heart Rate: > 120 bpm or < 50 bpm
Difference HR at Vx (increase or decrease) compared to pre-treatment > 30 bpm

ULNR = Upper Limit of Normal range

Appendix F Bronchoscopy Manual

Bronchoscopy Manual for clinical trial RO-2455-402-RD

Overview

Bronchoscopy will be performed according to the established protocol at each site.

Bronchoscopy will be performed for all randomized patients at V2 (randomization visit) and V6 (subsequent to 16 weeks of treatment) according to the American Thoracic Society and/or European Respiratory Society standards and will follow the specific procedures established at site. The biopsy procedure should be conducted at a minimum of 14 days after the sputum sample collection at visit V1 and V5.

The procedure will be deferred if the responsible investigator considers the Subject as unsuitable for bronchoscopy. The allowed frame for the adjournment is described in the protocol, Appendix A. For V0 – V2 the allowed time window is 6 weeks -3/+5 days and for V2 – V6 it is 16 weeks -7/+7 days.

In order to ensure good quality of biopsied material all sites will be requested to provide tissue samples (2-3 lobar bronchus and 2-3 sub-segmental airways specimens per Subject) harvested from the first three enrolled Subjects, which are expected to be recruited within 3 months from site initiation. These samples will be shipped to the Chief Pathologist of the trial in Padova (address see further below). Sections will be used to clarify the quality of the biopsy procedure conducted on the respective site.

Only after the biopsy samples are deemed to be of sufficient good quality by the central reader(s), will the bronchoscopist at the site be allowed to randomize further Subjects into the trial. The limit of 3 patients will be extended by another 3 patients in case the quality of one or more of the first samples is not sufficient and to give the site a chance to improve the sampling technique. In very exceptional cases and if the reason for failure is outside of the control of the site a further extension by additional 2 patients will be allowed. The site will receive a written confirmation with the approval to randomize further patients in the trial.

Subject preparation and procedure

An experienced bronchoscopist in a dedicated bronchoscopy unit will perform the procedure with facilities for the management of medical emergencies and resuscitation. The bronchoscopy and biopsy has to be performed according to the American Thoracic Society and/or European Respiratory Society standards and will follow the specific procedures established at site.

Bronchoscopies will be performed at each site. If possible a bronchoscope with a large diameter is preferred.

Disposable biopsy forceps provided by Takeda can be used for each Subject.

During the biopsy procedure a Biopsy Collection Worksheet (see Appendix G) must be completed. The top copy of this completed worksheet must be sent with the samples via courier to

laboratory for analyses. The second copy should be retained at the investigational site and filed in the Case Report Form (CRF) of the Subject.

Procedure of bronchoscopy

Sites should follow their standard hospital procedures for the medical and nursing care of the Subjects during and following the bronchoscopy.

All patients will be given a 24 h emergency contact number and will be followed up by phone within 24-48 h by the Investigator. Additionally a safety visit (visit V3 and V7) will be performed within 2 weeks, during which the respective Investigator will see the Subjects after each bronchoscopy session.

All medication administered at the time of bronchoscopy must be recorded in the Case Report Form (CRF) on the concomitant medication form.

Any adverse events (AEs) relating to the bronchoscopy must also be documented in the CRF on the Adverse Event form. Any serious adverse event (SAE) that occurs during or following the bronchoscopy, e.g. severe bleeding, pneumothorax or necessity for an overnight stay in hospital must be reported within 24 hours of occurrence by completing a SAE form and send to Takeda (for details, see Section 10.2.2 in the trial protocol).

Procedure of biopsy

Six endobronchial biopsies of adequate size will be taken from two airway generations (level). Biopsies from the lobar and sub segmental carinae areal will be collected.

On each biopsy occasion the biopsy size and overall quality (free of blood) should be macroscopically inspected by the bronchoscopist/assistant.

As two airway levels (i.e. lobar carinae and subsegmental carinae) are biopsied, the biopsies from these two sites should be separately recorded i.e. those from the lobar carinae labelled with the prefix A and those from the subsegmental carinae region labelled with the prefix B. For these two levels there should be 2-3 good quality biopsies for each site. The biopsies should be immediately placed in fixative. To avoid mixing of samples a plastic specimen should be used for each tissue sample.

The following information must be written on this plastic specimen using a histology pen;

- The trial ID (i.e. RO-2455-402-RD) and the CRF number
- The airway level (A or B)
- The visit number and date
- The date and time the biopsy placed in fixative.

The second set of biopsies at visit V6 should be taken from the same airway level (i.e. lobar carinae and subsegmental carinae) but from a the other side of the lung as compared to V2 biopsies..

If, for technical reasons, the bronchoscopy has to be terminated earlier than expected, documentation with information about the reason must be entered in the CRF page.

Fixing of the biopsy samples

Sites should follow standardized procedures for the fixing and embedding the biopsy sample in a paraffin wax block. For the immunohistochemical analyses in the laboratory of it is mandatory to get samples fixed in formaldehyde PBS solutions for four hours. All other steps including embedding in wax should be performed in the pathology laboratory of each investigational site following the standardized procedure as reported in the Biopsy Laboratory Manual (see Appendix G provided from laboratory.

Shipment of the samples

All biopsy samples of the Subjects from the RO-2455-402-RD trial will be sent for analysis in a paraffin block to:

Dipartimento di Scienze Cardiologiche, Toraciche e Vascolari
Department of Cardiac Thoracic and Vascular Sciences

The “Biopsy Collection Worksheet” and “Biopsy Processing Worksheet” must be attached to this shipment (see Appendix G).

Appendix G Biopsy Laboratory Manual
Daxas® Biopsy Trial RO-2455-402-RD

Laboratory Manual
Biopsy techniques:
Methodologies for immunohistochemistry

High Specialty Centre for Research and Care on Asthma and COPD

Department of Cardiac, Thoracic and Vascular Sciences
University of Padua
and
Padova City Hospital

1 INTRODUCTION 104
2 PROCESSING OF THE BIOPSY SAMPLES ON EACH INVESTIGATIONAL SITE 106
2.1 Formalin Fixation.....106
2.2 Paraffin Embedding 106
2.3 Step by step procedure to be followed on each investigational site 107
2.4 Criteria to maximize biopsy quality.....108
3 PROCESSING OF THE BIOPSY SAMPLES FOR ANALYSIS IN PROFESSOR SAETTA'S LABORATORY 108
3.1 Cutting.....108
3.2 Immunohistochemistry 109
3.2.1 Direct immunohistochemistry.....109
3.2.2 Indirect immunohistochemistry 110
3.3 Positive and negative controls 112
3.4 Quantification by light microscopy 113
4 REFERENCES 114

APPENDICES

Biopsy Collection Worksheet 116
Biopsy Processing Worksheet.....118

1 INTRODUCTION

Fiberoptic bronchoscopy provides a good tool to investigate bronchial biopsies, transbronchial biopsies, and bronchoalveolar lavage (BAL) in chronic inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) (1-5). The advantage of bronchial biopsies over other sampling techniques, such as induced sputum or BAL, is that they give anatomical information on airway morphology, therefore allowing the examination of the different compartments of the bronchial wall such as epithelium, subepithelium, smooth muscle and glands. Bronchial biopsies can be processed for light microscopy, electron microscopy and also for cell culture and cell cloning (6) or for detection of gene expression by quantitative polymerase chain reaction (PCR) (7,8). Electron microscopy allows analysis of cell ultrastructure but processing samples for this technique may be complicated and the amount of tissue examined is very small (6). In view of these disadvantages, light microscopy is the mostly widely used technique for the evaluation of airway biopsies (9). There are various methods of processing biopsy samples for light microscopic analysis: snap-freezing without prior fixation, paraformaldehyde fixation and freezing, formalin fixation and paraffin-embedding, fixation and glycol-methacrylate (GMA)-embedding. Each different method to process bronchial biopsies has advantages and disadvantages (see Table l). Snap-freezing without prior fixation provides samples for immunohistochemical analysis of most antigens, which are not masked by fixation. A disadvantage of this technique is that tissue structure is not well-preserved for morphometric analysis (9). Immediate fixation in freshly prepared paraformaldehyde and subsequent freezing has also been recommended, especially for specific techniques such as molecular analysis in situ. Antigen reactivity is relatively preserved; however, not all antigens will react to immunohistochemical analysis and the morphological preservation is suboptimal. The major advantage of formalin-fixation/paraffin embedding is that it provides excellent morphology, thus allowing a preliminary overview of the airway pathology using histochemical methods. A disadvantage of the technique is that formalin denatures proteins, by reacting primarily with basic aminoacids of the epitope, thus forming cross linking "methylene bridges". Therefore, formalin fixation will mask some antigens which cannot be easily revealed by immunohistochemical analysis (9). Several methods have been introduced for antigen retrieval such as digestion with proteolytic enzymes, e.g. trypsin (10), microwaving (11,12) or autoclaving (13). The advantage of GMA-embedding technique is the relative section thinness (1-2 µm), which gives a much greater resolution compared with a 5- µm thick section. This is because of thin sections having fewer focal planes through which the light microscope must focus. In addition, using thin sections, it is possible to do more than one cut through a single cell. This enables staining of two distinct epitopes on the same cells in adjacent sections. However, the fixation and resin embedding may significantly impact on antigen reactivity and cutting of ultrathin sections may be complicated and require expensive instruments (9).

TABLE 1: ***Advantages and disadvantages of different techniques for processing bronchial biopsies for light microscopic analysis***

Procedure	**Advantages**	**Disadvantages**
Snap-freezing without prior fixation	• immunoreactivity for all antibodies	• bad morphology • limited duration of immunoreactivity
Paraformaldehyde fixation and freezing	• reasonably good morphology • immunoreactivity for increasing number of antibodies	• not all antibodies are reacting
Formalin fixation and paraffin-embedding	• excellent morphology • immunoreactivity for increasing number of antibodies • routinely performed in pathology laboratories • inexpensive	• not all antibodies are reacting
Fixation and GMA-embedding	• excellent morphology • very thin sections • immunoreactivity for increasing number of antibodies	• not all antibodies are reacting • expensive instruments for cutting ultrathin sections.

Therefore, by far the most commonly employed method for processing tissue samples is formalin-fixation/paraffin embedding. This technique is routinely performed in pathology laboratories, and therefore universally available, is relatively inexpensive, samples are easily stored and can also easily travel from a site to another, which is an important issue in multicenter studies. Therefore, in the following paragraphs we will discuss in detail the different phases of processing airway biopsy samples for paraffin embedding (9).

2 PROCESSING OF THE BIOPSY SAMPLES ON EACH INVESTIGATIONAL SITE

2.1 Formalin Fixation

Since the autolytic process begins immediately, it is important to place the biopsy directly in the fixative. Endobronchial biopsy samples should not be placed in sterile phosphate-buffered saline as an interim holding procedure but must be fixed immediately following broncoscopy (14-15). The most commonly employed fixative is formalin (in common practice a formaldehyde solution approximately 4% in phosphate-buffered saline, see “Step by Step procedure” paragraph below) (9, 14-15). The volume of fixative should be large enough for complete penetration of formalin (approximately 10 ml in a 20 ml polyethylene-vial). Fixation time is a crucial factor for the quality of both histochemical and immunohistochemical analyses. It is important to balance between the need to fix enough for obtaining good morphology, but not fixing too much to prevent excessive antigen masking (14-15). Based on the average size of airway biopsies the ideal time for their fixation is 4 hours.

2.2 Paraffin Embedding

Following fixation, the process of embedding in paraffin wax requires exposing the biopsies to a series of chemicals for dehydration and clarification. For biopsy dehydration it is common use to pass them through a graded series of ethanol (see “Step by Step procedure” paragraph below) (9). The final step (100% ethanol) is crucial for complete dehydration; rather than using large volumes of alcohol it is important to change it often. Subsequently, biopsy clarification, through serial steps of a safety-approved clearing medium is needed to check that the process of dehydration has been successfully performed and that biopsies are ready to be included (see “Step by Step procedure” paragraph below) (9, 14-15). Paraffin is the most commonly employed medium for inclusion, is inexpensive and universally available, but it is essential that the paraffin quality is proven for histology (free of contaminating agents, with a controlled solidification point). Samples should first be infiltrated by liquid paraffin wax for an appropriate time to ensure complete penetration deep into the tissue and to avoid non-homogeneous embedding that will affect cutting (14-15). Once the tissue sample has been infiltrated in a tissue base mould, it is covered with a tissue cassette as a support for cutting and to allow hardening at room temperature (see “Step by Step procedure” paragraph below). Paraffin-embedded samples with this procedure may be stored for long periods in storage facilities that are universally available (no need for special storage facilities). Moreover, there are no particular technical hitches during transport of samples between laboratories.

For shipment details see Central Laboratory Manual.

2.3 Step by step procedure to be followed on each investigational site

STEPS:

1. Prepare 10% formalin, by adding 100 ml of 37% formaldehyde solution to 900 ml of Phosphate buffered saline (**PBS)** at pH 7.4. Mix thoroughly. This solution can be stored for a short period at 4°C (but not more than 1 month).
2. Prepare 70% ethanol by adding 70-ml absolute ethanol to 30-ml distilled water and mix. This solution can be stored at room temperature.
3. Prepare 90% ethanol by adding 90-ml absolute ethanol to 10-ml distilled water and mix. This solution can be stored at room temperature.
4. On the day of bronchoscopy melt paraffin wax in a stove at 58°C. This should be done in advance to assure that paraffin is liquid at the moment of inclusion.
5. Place biopsies in fixative (10% formalin) for 4 hours at room temperature. In this and in the following steps use 10 ml of liquid volume in 20-ml polyethylene-vials (acid and basic resistant). **Endobronchial biopsies should not be placed in sterile phosphate buffer saline (PBS) as an intermediate step, but they should be immediately placed in fixative.**
6. After fixation, dehydrate the biopsies by passing them through a graded series of ethanol at room temperature following this schedule:

70% ethanol	15 minutes
90% ethanol	15 minutes
Absolute ethanol	15 minutes
Absolute ethanol	15 minutes
Absolute ethanol	15 minutes

7. After dehydration, biopsies need to be clarified, through serial steps of a safety-approved clearing medium, following this schedule:

Clearing reagent	15 minutes
Clearing reagent	15 minutes
Clearing reagent	15 minutes

8. Place biopsies in liquid paraffin wax at 58°C for 1 hr (use ultrapure paraffin suitable for immune histology, e.g. Histotec by MERCK).
9. Place each biopsy in a tissue base mold containing liquid paraffin, cover with a tissue cassette previously marked with essential information and add liquid paraffin.
10. Allow to harden at room temperature.

11. Remove paraffin-embedded biopsies from the tissue base mold and store with desiccant in plastic bags in temperature-controlled facilities.

Summary

STEP	TEMPERATURE	TIME
Fixative(10%buffered formalin)	Room temperature	4 hours
70% alcohol(ethanol)	Room temperature	15 minutes
90% alcohol (ethanol)	Room temperature	15 minutes
Absolute ethanol	Room temperature	15 minutes
Absolute ethanol	Room temperature	15 minutes
Absolute ethanol	Room temperature	15 minutes
Clearing reagent	Room temperature	15 minutes
Clearing reagent	Room temperature	15 minutes
Clearing reagent	Room temperature	15 minutes
Paraffin wax	60 °C	60 minutes

2.4 Criteria to maximize biopsy quality

All possible efforts should be made to have samples of the maximum quality. The most important recommendation is to place biopsies in fixative immediately after collection. Endobronchial biopsies should not be placed in sterile phosphate buffer saline (PBS) as an intermediate step (14).

Furthermore, it is recommended that an assistant be present at the bronchoscopy to check that the endobronchial biopsy is of adequate macroscopic size and quality (no blood contamination). This evaluation step will maximize the yield of subsequent microscopic analysis. In fact, for the purpose of inflammatory cell assessment an evaluable biopsy should contain a large area of subepithelium excluding cartilage, glands or smooth muscle and, obviously crush artefacts and blood clots. In addition it is recommended that multiple biopsies are collected (4 to 6), to account for between-airway variability and to increase the possibility of having adequate samples. The minimal requirement is to have at least 1mm of basement membrane length (corresponding to an area of 0.1mm^2) evaluated for each patient. This is achieved by counting multiple biopsies (each one should contain about 0.3-0.5 mm of basement membrane length).

Rapid processing of samples within a quality-assessment pilot study allows feedback as to the adequacy of the biopsy technique which, in turn, permits improvements to be made during the course of the study.

3 PROCESSING OF THE BIOPSY SAMPLES FOR ANALYSIS IN PROFESSOR SAETTA'S LABORATORY

3.1 Cutting

The importance of consistent sectioning is also not to be overlooked. For routine cutting, a sled or rotary microtome can be used to cut a ribbon of 5 µm sections from formalin blocks (14-15). The solidified block of paraffin wax is fixed in a holder on the chuck of the microtome. The microtome

allows a reproducible forward advancement of the block towards the knife that will slice a section from the surface of the block. As sections are cut, the lower edge of the block melts slightly allowing this edge to join with the trailing edge of the previous section thus forming a ribbon of sections (15). Such ribbons of sections are floated on a thermostatic bath (water temperature should be at least 10°C below the wax melting point) to allow complete expansion of the sections. Individual sections are then gently split from the ribbon and picked up on glass slides that are usually coated to enhance adherence. Commercially available slides include those with positive charges (that attracts the negative charges of tissue proteins) or those prepared with adherent material (i.e. albumin or lysine, either of which will provide a sticky surface). Sections that are not flat and have non-adherent ridges will likely be digested or torn-off the slide during subsequent processing.

Sections should be drained thoroughly before storage (possibly in air-dry heaters at 37°C overnight). When possible, it is preferable to use freshly cut sections for immunohistochemistry, although they can be stored in a sealed container with desiccant.

To be processed for subsequent histochemical and immunohistochemical analyses slides must first undergo steps for deparaffìnation and rehydration.

3.2 Immunohistochemistry

Immunohistochemistry has been used widely to identify cell types and their subsets, markers of activation, cytokines and chemokines, and a variety of other tissue components. This technique has greatly facilitated cell phenotyping for both diagnosis and research. The method is based on the specific binding of an high-affinity antibody to the corresponding epitope, which is the smaller sequence of an antigen (usually about 5 to 10 amino acids) required for antibody binding. In principle, the technique comprises two main steps. In the first step, a primary antibody is applied, which binds specifically to the epitope to be detected in the tissue. In the second step, the antigen/antibody complex is visualized with direct or indirect detection techniques (8-9, 14-15).

Due to use of cross-linking fixatives that may mask antigen detection by immunohistochemistry, antigen retrieval techniques may be required prior to antibody binding. Several techniques have been introduced for antigen retrieval which make use of either chemical or physical methods (16-18). The most common are digestion with proteolytic enzymes (e.g. trypsin), microwaving or autoclaving. To achieve the optimal protocol for each specific antigen, it is common practice to standardize in set-up experiments the different variables (e.g. method of heating, temperature, time, pH).

3.2.1 Direct immunohistochemistry

In the direct method for immunoenzymatic staining, an enzyme-labelled primary antibody reacts with the antigen in the biopsy section. Subsequently, the antibody binding is detected by the presence of a chromogenic reaction product resulting from the action of the enzyme on specific substrates. This method utilizes only a single antibody: therefore it can be completed rapidly, and non-specific reactions are minimized. However since staining involves only a single complex between the antigen and the labelled antibody, the signal is often relatively weak. This technique is

now used only rarely and has been replaced by indirect methods which include soluble enzyme immune-complexes and avidin-biotin methods (8).

Figure 1: Schematic representation of direct immunohistochemistry

3.2.2 Indirect immunohistochemistry

This technique most widely uses methods involving soluble-enzyme immune-complexes or avidin/biotin complexes. Soluble-enzyme immune-complexes techniques usually use alkaline phosphatase-anti alkaline phosphatase complex (APAAP) method or the peroxidase-antiperoxidase complex (PAP). The method used for analysis of biopsy samples in Daxas® Biopsy Trial RO-2455-402-RD will be the APAAP method with Fast Red as a chromogen, so that positive cells will be identified by a red coloured end-reaction product. The technique involves the following steps:

1. **Blocking of nonspecific binding sites.** This step aims to eliminate nonspecific reactivity by saturation of binding sites and is performed by incubating the sections with normal serum. This step and the following ones (primary and secondary antibodies) are usually performed in humidified chambers to avoid sections dry out. When the detection system utilizes peroxidase as a substrate, prior to addition of the primary antibody a treatment with sodium azide or hydrogen peroxide is required to inhibit endogenous peroxidise activity (8).
2. **Incubation with unconjugated primary antibody**. The primary monoclonal or polyclonal antibody is applied to the sections at optimal dilutions (which are determined by initial titration). This step is usually performed for 30 min to 1hour at room temperature, but incubation time may be increased (for instance overnight at 4°C). Subsequently, unbound primary Ab is removed by washing. An irrelevant antibody of the same isotype should be used as a negative "control" in each immunohistochemical run. There are numerous advantages of monoclonal antibodies in immunohistochemistry over their polyclonal counterparts; these include high homogeneity, absence of nonspecific reactions, and negligible lot-to-lot variability (8).
3. **Incubation with secondary antibody/enzyme immune complex.** A secondary antibody, directed against immunoglobulins from the species used for the primary antibody, is applied to the sections (usually for 30 min at room temperature). This antibody, or link Ab, is added in excess, so one of its two binding sites (Fab sites) remains free. Unbound link Ab is then removed by thorough washing. In a third step, the enzyme-immune-complex is added, consisting of an enzyme (usually phosphatase or peroxidase) conjugated to an

antibody. In this step, the second free binding site of the "link Ab" will bind the enzyme-immune-complex (usually during a 30 min period) (Figure 2). With most recent systems the second and third steps (link antibody and enzyme-immune-complexes) have been merged in a single step that utilizes an enzyme-conjugated secondary-antibody; this allows a more rapid procedure assuring adequate signal amplification. Soluble immune complex methods include the alkaline phosphatase-anti alkaline phosphatase complex (APAAP) method and the peroxidase-antiperoxidase complex (PAP) method (8).

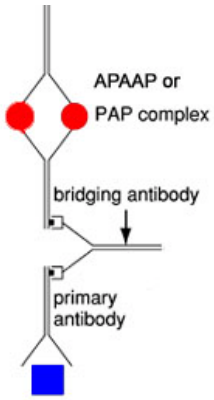

Figure 2. Schematic representation of the immunoenzymatic staining method using soluble enzyme immune complex methods. PAP: peroxidase-antiperoxidase complex. APAAP: alkaline phosphatase anti-alkaline phosphatase complex.

1. **Incubation with a substrate solution**. Finally, Ab binding is detected by the presence of a coloured end-reaction product resulting from interaction of the enzyme (peroxidase or alkaline phosphatase) with its specific substrate. Fast red is commonly used as chromogen in immunohistochemical staining procedures utilizing alkaline phosphatase. To minimize the background staining due to endogenous alkaline phosphatase, addition of levamisole could be helpful. Conversely, in staining procedures utilizing peroxidase, diaminobenzidine (DAB) which yields a brown reaction product, or 3-amino-9-ethyl-carbazole (AEC) which yields a red product, can be used (8).
2. **Counterstaining.** Sections are then counterstained with haematoxylin or neutral red, nuclear fast red or light green, washed in tap water and mounted in an aqueous mounting medium, or dehydrated and mounted in a suitable mounting medium. Fast red and 3-amino-9-ethyl-carbazole form an end-reaction product which is soluble in organic compounds (such as alcohols). Therefore, it is necessary to use a non-alcoholic counterstain (such as Carazzi's or Mayer's haematoxylin) and an aqueous based mounting medium.

An alternative method for indirect immunohistochemistry is based on ***avidin-biotin complexes*** (Figure 3). The avidin-biotin technique utilizes the high affinity of avidin or streptavidin for biotin. In this method the general procedure differs from that described above in respect of the following: l) a secondary biotinylated monoclonal Ab is applied to the sections. In this case an excess of

biotinylated antibody is unnecessary as free Fab sites are not required for binding the preformed avidin-biotin enzyme complex (ABComplex); 2) the preformed ABComplex consists of a solution in an optimal ratio of biotinylated enzyme (alkaline phosphatase or peroxidase) to avidin (ABComplex) or streptavidin (StreptABComplex). When the ABComplex or the Strept-ABComplex is applied to the sections they will bind strongly to the biotinylated antibody. As with the previous methods, before incubation with primary Ab, endogenous avidin biotin-binding activity should be blocked by treating the sections first with avidin and then with biotin (8).

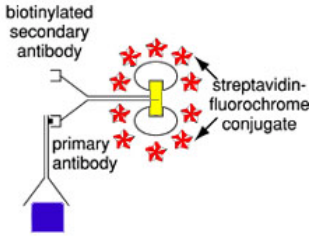

Figure 3: Schematic representation of the immunoenzymatic staining method using avidin·biotin methods.

Immunohistochemistry applied to biopsy sections is usually performed using immunoenzymatic staining methods which give resultant coloured end-reaction products. Other methods of visualization such as fluorescence can also be applied, but tissue morphology is not easily visible without phase contrast microscopy and the fluorochromes are usually short-lived. However, a distinct advantage of the fluorescence technique is that double-labelling methods can be applied where red and green fluorochromes may be separately seen using selected filter blocks. By a procedure of photographic double exposures, those cells which are double-labelled appear in the resultant mixture of colour (i.e. yellow) (8).

3.3 Positive and negative controls

For all the immunohistochemical procedures listed above, appropriate positive and negative controls must be included in each staining run. As positive controls, use can be made of tissue sections known to be positive for the antigen under study: tonsil or clones producing specific cytokines or cells transfected with Cdna encoding specific human cytokines. This method can also be implemented to assess the specificity of the primary Ab. To confirm the specificity, use can be made of tissue sections known to be negative for the antigen under study. Affinity absorption of the primary Ab with highly purified antigen provides the ideal negative control for differentiating specific from nonspecific staining. As negative controls, use can be made of sections of the tissue under study incubated either without primary monoclonal Ab or with isotype and species matched irrelevant primary monoclonal Abs (8, 14).

3.4 Quantification by light microscopy

Light microscopic analysis can be performed either directly using an appropriate magnification (400-1.000X) or by recording the image and making the assessment with the aid of a computerized image analysis system. It is recommended that between two and five sections, possibly from different biopsies are selected, appropriately stained and counted for each cell phenotype. The reasons for this include clustering of inflammatory cells, non-normality of the data distribution for counts of such cells and sometimes infrequency of the cell type of interest. The intra- and the interobserver reproducibility need to be assessed. These are usually computed using the coefficient of variation and/or the intraclass coefficient for repeated measurements among at least three replicate measurements of morphometric parameters performed by the same observer or different observers. Alternatively, other tests that can be used for estimating inter-rater agreement may include a simple regression or the more accurate Bland-Altman test (8,14-15).

Inflammatory cells are usually assessed in the area beneath the epithelial basement membrane (referred to as subepithelium or lamina propria), but they may be evaluated in the epithelium as well, provided it is well preserved and not damaged. Both in the epithelium and in the subepithelium inflammatory cells are counted in several nonoverlapping high power fields until all the available area is covered. The depth of the subepithelial zone examined needs to be standardized in order to have comparable data in different subjects. In the literature, the depths chosen range from 50 to 200 μm, but cells are most commonly evaluated in the area 100 μm below the basement membrane. Performing cell counts in this area is preferable to the alternative technique of performing them in the whole tissue available, since this minimize heterogeneity. Moreover, cell densities are usually higher in the area immediately beneath the basement membrane, while the analysis of the whole tissue (including areas with lower cell density) usually dilutes the message. The areas occupied by bronchial smooth muscle and mucus-secreting glands should not be included in the total area assessed, otherwise variations in the areas of these structures will seriously alter the results (8, 14).

The final result is expressed as number of cells per area of tissue examined or as number of cells per mm length of basement membrane (especially for epithelial counts). A single value is obtained from the average of the measurements performed in each section for a given subject.

Since the probability of visible cells being counted in a two-dimensional section is proportional to their size, density, and orientation, this may potentially introduce a bias in favour of the larger cut profiles (14). However, the size variability for each cell phenotype is limited (while there is larger variability among different cell types). Moreover, since nuclear size varies less than overall cell size, a good option is opting to count only cells whose nucleus appears in the plane of the tissue section, thus reducing this bias. Ideally, application of stereology and assessment of the cell “volume density” could be applied (14). However, this has not been commonly performed in the literature in studies assessing the effect of treatment on biopsy inflammation and therefore results would be difficult to compare with those of previous studies.

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Biopsy Collection Worksheet

(to be filled in the bronchoscopy facility)

Date…… Visit Number……. CRF nr……

Name of Bronchoscopist: ……. ……

<table>
<tr><th colspan="3"></th><th>Macroscopic biopsy quality*</th></tr>
<tr><td rowspan="6">Total number of biopsies collected (Nr…….....)

From which anatomical lobe
(e.g. dx, superior):
…….
……..</td><td rowspan="3">Lobar (A)
Nr…….</td><td>A1 □</td><td>……..
……..</td></tr>
<tr><td>A2 □</td><td>……..
……..</td></tr>
<tr><td>A3 □</td><td>……..
……..</td></tr>
<tr><td rowspan="3">Sub-Segmental (B)
Nr……..</td><td>B1 □</td><td>……..
……..</td></tr>
<tr><td>B2 □</td><td>……..
……..</td></tr>
<tr><td>B3 □</td><td>……..
……..</td></tr>
</table>

The following information **must** be written on each biopsy vial using a histology pen:

- The CRF number, visit number and date
- The airway level and consecutive number (e.g. A1, A2, A3, B1, B2, B3)
- The time biopsy was placed in fixative.

Mandatory: Please specify date and time of biopsy placed in fixative……

ADDITIONAL NOTES ……

……

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……

……

* please note the macroscopic appearance (large, medium, small and presence/absence of blood)

Biopsy Processing Worksheet

(to be filled in the pathology facility)

Date…… Visit Number……. CRF nr……

Total number of biopsies received (nr…….....)	**A1** □	**A2** □	**A3** □
	B1 □	**B2** □	**B3** □

Please report date and time of biopsy placed in fixative ……...……..

Please report date and time of end fixation……..

(note: a time frame of 4 hours has to be adhered to, see also the respective lab manual)

Please report date and time of end inclusion……...……

Total number of biopsy included (nr……..)	**A1** □	**A2** □	**A3** □
	B1 □	**B2** □	**B3** □

The following information must be written on each biopsy cassette using a histology pen:

- The CRF number, visit number and date
- The airway level and consecutive number (e.g. A1, A2, A3, B1, B2, B3)

ADDITIONAL NOTES ……
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……
……
……
……

Appendix H Sputum Laboratory Manual

Manual Sputum Induction and Processing in COPD studies (Differential Cell Counts and Biomarkers)

INDUCED SPUTUM 121
1.1 Sputum Induction.....121
1.1.1 General Comments and Safety Aspects.....121
1.1.2 Stopping Criteria of the Sputum Induction Procedure.....121
1.1.3 Sputum Production.....121
1.1.4 Essential Equipment and Materials for Sputum Induction 122
1.2 Detailed Induction Procedure 122
1.3 Sputum Processing.....124
1.3.1 First step: Selection of Sputum Plugs 124
1.3.2 Rating of Sputum Quality 124
1.3.3 Essential Equipment and Materials for Sputum Processing 124
1.3.4 Detailed Processing Procedure 125
1.3.5 Giemsa Staining Method.....128

Induced Sputum

1.1 Sputum Induction

1.1.1 General Comments and Safety Aspects

Sputum induction will be restricted to patients with a minimum post-bronchodilator FEV_1 equal to or greater than 1 Liter and FEV_1 greater than 30% pred. prior to every sputum induction and must be obtained according to ATS/ERS criteria. In case sputum induction will not be performed within two hours of administration of the bronchodilator for lung function assessments, two additional puffs of salbutamol (2 x 100 µg) will be administered before sputum induction and the best FEV_1 will be used as baseline (ATS/ERS criteria to be followed).

The procedure of sputum induction must be conducted by an experienced and trained technician. The responsible physician must be present on site during induction. Appropriate emergency equipment must be available.

After completion of the whole sputum induction, the patient will remain in the unit until FEV_1 has returned to within 10% of baseline.

Concerning the cleaning of surfaces and mouthpieces, as well as maintenance and technical safety of the equipment, please refer to local hygiene and safety requirements.

Concerning the handling of substances, which are needed during processing of sputum samples (e.g. sputolysin, methanol, acetone), please refer to the universally valid safety data sheets of these products.

1.1.2 Stopping Criteria of the Sputum Induction Procedure

Stopping criteria are as follows:

a) Patient reports about bothersome symptoms or

b) A fall of ≥20% in FEV_1 compared to the post-bronchodilator value occurs or

c) The responsible physician decides to discontinue the induction due to other safety reasons.

1.1.3 Sputum Production

Inform the patient about the need to collect the sputum as uncontaminated as possible and that sputum are secretions from the lower respiratory tract and not from the nose (post nasal secretions). Ask the patient to thoroughly blow their nose, to intensively rinse their mouth, to swallow some water and to clean their throat. Watch this procedure. Ask the patient to cough up sputum. Often it is easier to ask the patient to cuff up intensively (forced expiration maneuver, which sometimes brings up sputum far enough to be expectorated by light coughing).

The expectorate should be given directly into a clear plastic dish, where the selection process will be performed.

1.1.4 Essential Equipment and Materials for Sputum Induction

- Refrigerator, to store saline solutions and sputum samples
- Ultrasonic nebulizer (NE-U12, Omron, Tokyo, Japan/MMD – Mass Median Diameter: 4.9 µm, mean output: 1.72 mL/min or equivalent)
- Salbutamol MDI 100 µg (e.g. Sultanol® 100 µg)
- Spirometer
- Emergency equipment
- Saline solutions 0.9% and 3%
- Buy ready to use or dissolve 0.9% NaCl, 3% NaCl p.a. in 100 mL sterile water. Store at 4°C
- Nose clips
- Microscope
- Cups or dishes for sputum production and selection of plugs
- Pipettes, forceps, inoculation loops
- Stopwatch
- Eppendorf-cups and 15 mL tubes with known weight
- Disposable cups, drinking water, paper tissues
- Cleaning material

1.2 Detailed Induction Procedure

The aim is to assist the sputum production by inhaling nebulized saline (0.9% and 3%) for a total time of 20 minutes. Be aware of the stopping criteria listed above.

1. Inform patient about procedure and potential side effects, which should be indicated immediately by the patient (cough, chest tightness, burning sensation in the throat by saline) so that the physician can decide whether the induction can be continued or not.
2. Set up the nebulizer, add 30 ml of 0.9% saline, attach hoses, mouthpiece.
3. Skip step 3-5 if reversibility testing (e.g. 400 µg of Salbutamol) or bronchodilator testing (e.g. 400 µg of Salbutamol and 80 µg of Ipratropiumbromid) was done < 2 hours before start of sputum induction and document the best post-bronchodilator FEV_1 as the baseline FEV_1. This baseline value will be the reference value for the calculation of the decline in FEV_1 during sputum induction.
4. Measure and document pre-bronchodilator FEV_1 (according to ATS/ERS guidelines).

5. Administer 200 µg of Salbutamol per MDI or perform the induction after the bronchodilator test on the respective trial day as outlined in the protocol.
6. After waiting for 10 to 20 minutes, measure pulmonary function again (according to ATS/ERS guidelines) and document the best postbronchodilator FEV_1. This value will be the baseline FEV1 used as reference value for the calculation of the decline in FEV_1 during sputum induction.
7. Calculate 10% and 20% decline of the baseline FEV_1.
8. Start the inhalation periods.

Do not forget to document collection time = start of first induction period. Every 5 minutes inhalation will be followed by pulmonary function test and the attempt of the patient to expectorate sputum.

Please remind the patient to perform the cleansing procedure (rinse mouth, clear throat, blow nose). The sequence of FEV_1 measurement and sputum production can be chosen by the patient.

The pulmonary function tests during sputum induction are done for safety reasons. In order to not exhaust the patient the FEV_1 measurements should be restricted to 2 attempts per inhalation period.

For the safety of the patients the decline of FEV_1 will be evaluated after each inhalation period.

Please check FEV_1 and compare the value with the reference FEV_1 (post-bronchodilator value):

- If the decline in FEV_1 compared to baseline FEV_1 is <10% start the next inhalation period.
- If the decline in FEV_1 compared to baseline FEV_1 is 10%-19% you can proceed further with the next inhalation period. In this case concentration of saline will not be increased.
- If the decline of FEV_1 is ≥20% the sputum induction will be stopped.

9. Start with the first inhalation period (0.9% NaCl) for 5 minutes. Patient should be wearing a nose clip and should maintain normal tidal breathing.
10. Measure FEV_1 after the inhalation period 2 times. Document the highest value on the sputum worksheet.
11. Collect the sputum according to the procedures and store the sputum sample at 4 - 8°C.
12. Continue with the second inhalation period (0.9% NaCl) for 5 minutes, Proceed as given in item 8-10.
13. Continue with the third inhalation period (3% NaCl) for 5 minutes and proceed as described under item 8-10.
14. Continue with the fourth inhalation period (3% NaCl) for 5 minutes and proceed as described under item 8 -10.

Note: If no sputum can be produced at any time during sputum induction procedure move on to the next inhalation step and avoid tiring the patient.

1.3 Sputum Processing

The sputum samples should be stored at 4 - 8°C and the processing should start directly after the last induction.

Do not forget to document the processing time (Start time of sputum plug selection).

1.3.1 First step: Selection of Sputum Plugs

A careful selection of sputum is crucial to obtain high quality sputum samples. The time invested here will be rewarded during the differential cell count.

The selection of sputum plugs should be performed in a petri dish.

There are a number of techniques to separate the "good" plugs (viscid and dense, mainly opaque parts) from the part of expectorate that mainly contains saliva. Use forceps, pipettes or other devices (inoculation loops) to pull the sputum out of the surrounding saliva. Pulling a plug across a clean and dry area of a dish sometimes helps to get rid of saliva.

The selected material should be checked by microscope to assure the absence (as far as possible) of squamous cells (irregular large cells) and the presence of lower respiratory tract cells (smaller cells with round shape) within plugs. There are sometimes very dense plug-like accumulations of squamous cells, which could be mistaken for sputum.

All selected material from the inhalation periods (if available) is pooled in one petri dish.

The selection should result in a representative sputum sample of at least 50 mg. Therefore, the more is collected, the better. BUT, if the collectable amount is too large (> 500 mg, difficulties in handling during processing) consider taking only an aliquot of the selected material. In addition, the time necessary for the selection procedure should remain within reasonable limits (e.g. some expectorate contains a lot of sputum, but very dissolved, without real plugs. Document this by a bad rating, and try to obtain as much material, as needed for the analysis).

1.3.2 Rating of Sputum Quality

The quality of the sputum samples should be estimated on a scale from 1 to 6, whereas 1 is the best note for a very good quality, a lot of easy to select sputum plugs and 6 is the note for a sputum with a lot of squamous cells and diffuse, hardly to select cells.

1.3.3 Essential Equipment and Materials for Sputum Processing

- Distilled water
- Trypan Blue (e.g. Sigma T8154)
- Bovine serum albumin, BSA (e.g. Sigma A2153)
- Sputolysin (= DTT, Calbiochem 560000)

- PBS (e.g. Gibco 10x 14200-067)
- Methanol p.a. (Merck)
- Bench rocker
- Timer
- Neubauer chamber
- Cytocentrifuge
- Precision balance
- Centrifuge
- Glass chambers for staining and fixation
- Reusable plastic funnel for filter
- Giemsa stain (or equivalent)
- Adjustable pipettes
- Adjustable vortexer
- Nylon mesh 50 µm for filtration (PA-50/37 Nylon (PA6.6), Franz Eckert GmbH)

1.3.4 Detailed Processing Procedure

1. Prepare Sputolysin (DTT) working solution by diluting the stock solution 1:10 with distilled water (e.g. 1 mL Sputolysin (DTT) stock + 9 mL distilled water) and mix thoroughly. Store the stock solution at room temperature. The working solution should be prepared fresh daily.
2. Determine the weight (volume) of the selected sputum plugs by weighing the tube and subtraction of the value of the empty tube.
3. Example: Sputum weight = 100 mg (100 µL)
4. Add 4 volumes of Sputolysin (DTT) working solution (Example: 400 µL)
5. Mix gently (by hand or slowly vortex) and incubate for 15 minutes at a bench rocker. This procedure should homogenize most sputum samples, but there are some samples in which the mucus is difficult to break down. After homogenization, add 4 volumes PBS to achieve a 9-fold final dilution of the sputum sample (Some small remaining undissolved plugs are frequently homogenized when the sample is gently mixed with PBS).

Example: Total volume after homogenization = 500 µL. 400 µL (4 times sputum volume of 100 µL) of PBS need to be added to the sample. Mix gently.

6. FILTRATION: Do not filter if total volume is <1 mL, proceed with unfiltered sample as described. Put a filter (50 µm nylon mesh), which has been pre-wetted with PBS, into the

funnel. Set the funnel into a pre-weighed conical tube and filter the cell solution through the nylon mesh. Record the total weight of the filtrate with the tube and subtract the weight of the empty tube to obtain the weight of the filtered cell solution. This way the loss of volume due to filtration can be estimated.

7. Centrifuge the homogenized and diluted sample at 600 g for 10 minutes at room temperature.
8. Take off the supernatant and disperse equally into labelled cryotubes.

Attention: Be careful to avoid aspiration of cells from the pellet. In case of aspiration, centrifuge sample once more.

The following information on the cryotube labels is mandatory:

– trial ID (i.e. RO-2455-402-RD)
– site/centre no.
– Subject ID
– visit no.
– visit date

Store them immediately until the end of the trial at -70°C to -80°C at each trial site.

Do not forget to document storage time!

Boxes containing the biomarker samples (including back-up samples) will be shipped on the sponsor's request.

Add PBS/BSA (1% BSA, 100 mg in 10 mL PBS, store at 4°C and check for contamination frequently) to the cell pellet and gently re-suspend. The amount of PBS/BSA depends on the size of the cell pellet. For a 100 mg sputum plug at the beginning about 200 µL of PBS/BSA is sufficient, but the amount should be increased to avoid excessive cell densities during cell and viability count. Weigh the cup or tube after addition of PBS/BSA and determine the total volume in which the cells are re-suspended. This step is critical for the calculation of "absolute cell counts".

Example: Added volume of PBS/BSA = 200 µL, total weight (cell pellet + any small amount of remaining supernatant + PBS/BSA) = 250 mg (250 µL)

9. Take an aliquot from the PBS/BSA cell mix (e.g. 10 µL) and add trypan blue solution (e.g. 10 µL). Mix, and fill the prepared Neubauer chamber with a sufficient amount (the time from adding trypan blue to the cells until determination of cellular viability should remain within certain time limits (a few minutes) and should be comparable between different samples).

Count all non-squamous cells within the given limits of the chamber and classify between dead (blue) and alive (here from at least 100 non-squamous cells).

Calculate the cell concentration (10^6/ml PBS/BSA mix), considering the dilution factor introduced by the trypan blue, and the cell viability.

Example:
Total number of cells (blue and alive) in one large square (16 small squares of the chamber) = 50 cells, cell count in two large squares is 100, 80 viable + 20 blue cells.

Cell count in the trypan blue mix = 50 x 10.000 (factor depending on volume in Neubauer chamber) = 500.000 cells / mL.

Cell count in the PBS/BSA cellmix = (500.000 x 20 µL)/10 µL = 1.000.000 = 1.0 x 106 cells/mL (PBS/BSA cellmix)

Viability: number of viable cells / total number of cells (dead + viable) x 100 (here: 80/100 x 100 = 80% viability)

10. The calculated cell count in the PBS/BSA cellmix will be needed to calculate the amount of mix needed to prepare a cytospin. 30.000 cells should be centrifuged upon a cytospin slide to optimize the amount of cells within 1 field of vision during differential cell count. Add 100 µL of adjusted cell mix (see below) to each funnel and (for the Shandon) centrifuge at 800 RPM for 5 minutes. Keep the rest of the PBS BSA mix (at 4°C) until you made sure that the cytospins turned out well (microscopic control of cell density and distribution).

Example:
Calculation for 30.000 cells: Cell concentration in the PBS/BSA cellmix = 1.000.000 cells / mL 30.000 cells / 1.000.000 cells/ mL= 0.030 mL 0.030 mL = 30 µL of the PBS/BSA cellmix are needed for 1 cytospin. For the Shandon cytocentrifuge 100 µL of cell mix should be added to the funnels, therefore fill up the 30 µL PBS/BSA cellmix with 70 µL PBS/BSA. Adjust volumes, depending on the number of cytospins needed (4 slides = 120 µL PBS/BSA mix + 280 µL PBS = 400µL).

NOTE: It has happened that the PBS/BSA cellmix starts gelation before a cytospin can be prepared. The mix becomes very difficult to pipette and has a gel like consistence. The reason for this is unknown, but it helps to wash the cells once or twice by adding PBS or PBS/BSA, centrifuge the sample again, and resuspend the pellet in PBS/BSA again. This sometimes happens during cytocentrifugation too, leaving a gel like mucus on the cytospin that takes “forever” to dry. Try washing the cells and prepare new cytospins. Some “sputologist” argue that this never happens after filtration of samples.

11. Prepare 4 cytospin slides. Stain 2 immediately as described below and leave 2 slides fixed and unstained as backup at site. All sputum slides should be clearly labelled.

The following information on the slides is mandatory:

– trial ID (i.e. RO-2455-402-RD)

– site/centre no.

– Subject ID

– visit no.

– visit date

12. Take out the cytospins, dry and fix as required (see below: Giemsa). Check if enough cells are on the slide and get a first impression on slide quality right away.

It's still time to make fresh cytospins (in case not sufficient or too many cells were used).

Giemsa stain according to the method recommended by the manufacturer for optimum results (see below). Each site is required to check the quality of the slides prior to sending them to the Sputum Laboratory / Central Laboratory.

1.3.5 Giemsa Staining Method

Sputum slides should be air-dried for 20 to 30 minutes, than be fixed for five minutes in methanol p.a.

Preparation of Giemsa-staining solution (must be freshly prepared each day.):

Buffer solution:

16 ml 0.5 M NaH2PO4 (=69 g/l) = 1.104 g in 16 ml

32 ml 0.5 M K2HPO4 (=87 g/l) = 2.784 g in 32 ml

+ 952 ml Aqua dest.

Adjust to pH 6.5 – 6.8 with 25% HCL (add about 24 drops HCL)

Buffer solution is stable at 4 – 8°C for 10 days

For Giemsa-staining solution filtrate about 10 mL Giemsa-stock solution and mix 10 mL filtrated Giemsa-solution with 170 mL buffer solution.

For staining take 60 mL of the fresh prepared Giemsa-staining solution in the glas cuvette. Stain the fixed Sputum slides for 15 minutes and rinse them afterwards with Aqua dest.

Alternatively you can use diff-quick staining kit ready to use (Fa. Fisher-Scientific) and proceed like described in the instruction manual.

Two slides together with a copy of the sputum worksheet of every patient need to be sent to the Sputum Laboratory / Central Laboratory. The two remaining slides will be a back-up and will be stored at the site until the end of the trial.

Slides of Screening visit should be sent immediately to the Sputum Laboratory / Central Laboratory (details for shipment will be listed in the Central Laboratory Manual).

Slides of all other visits should be sent as soon as slides of four visits are completed (does not need to be of the same patient) to the Sputum Laboratory at Pulmonary Research Institute (PRI) as well.

To:

Pneumologisches Forschungsinstitut am Krankenhaus Großhansdorf

For further questions please contact :

T:
Email:

Appendix I Microbiome sub study: The interaction between changes in the lung microbiota, airway inflammation and mucin production resulting from roflumilast therapy

1) INTRODUCTION

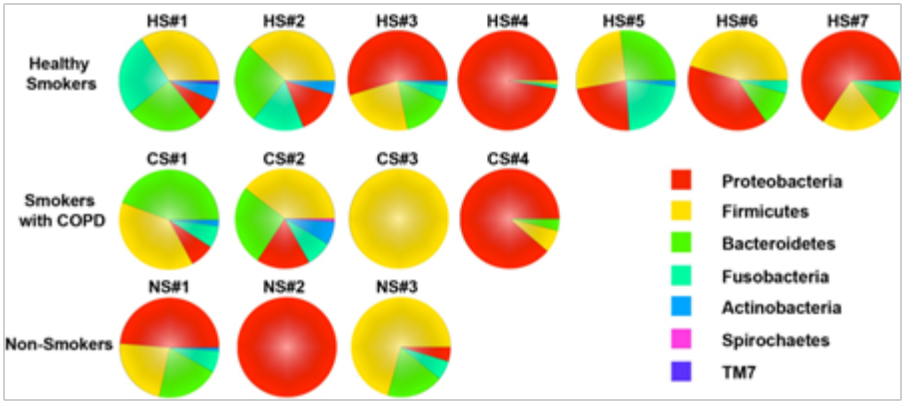
This appendix describes a sub study linked to **RO-2455-402-RD: Roflumilast bronchial biopsy**. COPD is a highly prevalent disorder characterized by airflow obstruction that is not completely reversible[1], and is one of few chronic disorders with rising mortality and morbidity. There are limited therapeutic options for COPD with few that alter disease course. COPD is an inflammatory disorder with an inflammatory/immune response that persists despite smoking cessation, varies by the patient population, the method of assessment, and the timing of measurement1. Furthermore, pulmonary and systemic inflammation intensify during an acute exacerbation of symptoms, an AECOPD[2]. The genesis of this inflammatory response remains unclear with theories including response to autoantigens or persistent or recurrent infection that may serve as a stimulator of specific immune responses or as a polyclonal activator.

Chronic bacterial colonization in COPD has been evaluated predominantly by culture-based methods; the magnitude and type of organisms varies widely[3]. The presence of bacteria in an organ that has always been considered sterile has been considered to be simply a side effect of the disease. Recent evidence, however, has called this belief into question. It is now apparent that humans are 'super-organisms' composed of not only of human cells, but also ten times that number of microbes inhabiting the skin and mucosal surfaces. Collectively, this is known as the human microbiome[4]. These microbes are important to health and have been identified as having roles in the control of allergy, eczema, obesity and resistance to enteric diseases. Many of these organisms are difficult or impossible to culture; however, advancements in culture-independent microbiology (using the bacterial 16S rRNA gene) and high-throughput sequencing has made it possible for entire metagenomes (genomic information from each of the organisms at a particular location) to be rapidly characterized.

These modern molecular techniques have been infrequently applied to characterize the lung microbiota. This approach has shown microbial diversity at least 100-1000 times greater than estimates based on culture. In cystic fibrosis, 16S-based surveys of respiratory samples have confirmed that a complex microbiota can be identified from the lung, and that culture underestimates its diversity. In cystic fibrosis sputa, thirteen bacterial species were identified by culture compared with 53 species using molecular techniques, including five putative new pathogenic species [5]. Similar diversity was recently noted by a separate group[6]. Bronchoscopic studies suggest that the lungs of healthy smokers are inhabited by diverse types of bacteria in relatively small numbers and that the composition of this microbiome changes with disease[7, 8]. In 16S rRNA amplicons of the sputum of poorly controlled severe asthmatics concentrations and bacterial diversity were significantly higher than in sputum of control subjects. Additionally, the relative abundance of particular phylotypes was highly correlated with the degree of bronchial hyper-responsiveness [9]. In 11 asthmatics with mild disease, five COPD subjects with moderate spirometric disease and 8 controls bronchoscopic and bronchoalveolar (BAL)-defined microbiota were similar[7]. In eight mechanically-ventilated subjects with an AECOPD a diverse microbial

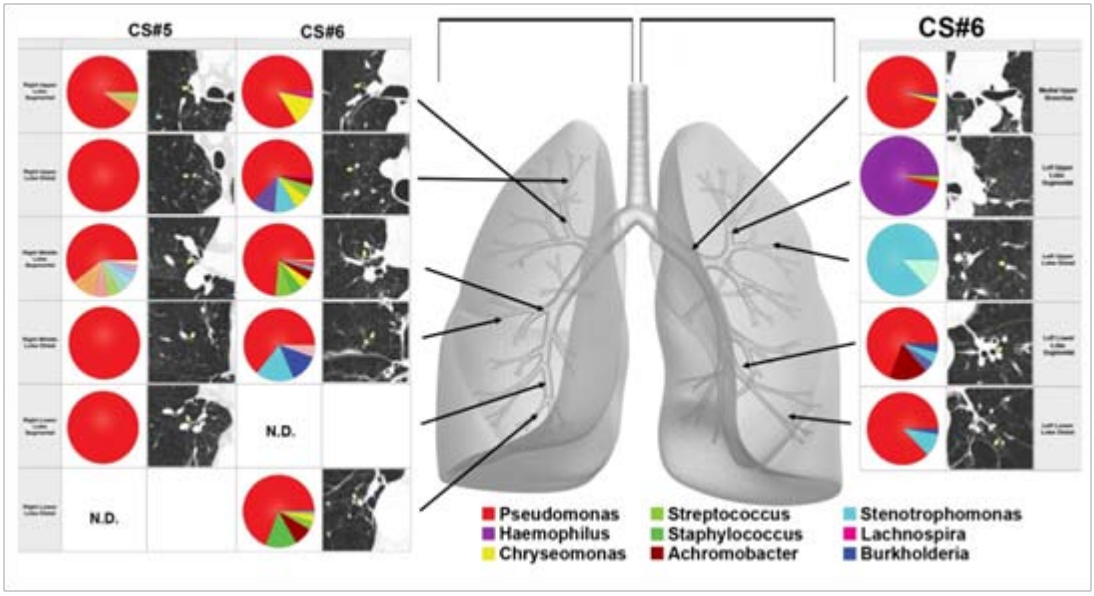
community was identified, despite recent or on-going antibiotic exposure [10]. Our recently published study extends these findings by suggesting that the respiratory tract of COPD patients harbours a complex microbial community during stable states (**Figure 1**)[8].

Figure 1. Taxonomic classification at the phylum level of BAL samples of healthy smokers (HS), COPD patients (CS) and nonsmokers with normal spirometry (NS) at a phylum level.



COPD is known to be a highly heterogeneous disease [11], with widely varying clinical symptoms, health status and disease progression. Furthermore, disease heterogeneity impacts both therapeutic approaches and our understanding of disease pathogenesis[11]. Distinct phenotypic groups include patients with predominance of cough and sputum production. Some COPD patients experience recurrent exacerbations with a threshold of > 2 events/year appearing to identify a 'frequent exacerbator' phenotype[12]. Computed tomographic imaging has identified patients with worse emphysema, who experience increased dyspnea, decreased health status and increased mortality[11]. Airway structure is similarly quite heterogeneous with increasing abnormality by CT associated with increased cough, breathlessness and worse health status. Our recent data also demonstrates that the lung has complex regional variations in microbiota (**Figure 2**); pseudomonas species are highly prevalent.

Figure 2. Regional heterogeneity is noted in lung microbiota. *Using post-operative explants multiple samples were harvested from the regions indicated by the arrows on the gray lung schematic in two COPD explants. Pie diagrams depict the genus level classification of 16S sequences, and the CT images demonstrate the absence of bronchiectasis in the airways adjacent to where samples were obtained. The key for the nine most abundant organisms is provided below the lung schematic. Significant heterogeneity is seen within the lungs from these COPD subjects.*



Previous data have suggested that smokers with symptoms of chronic bronchitis exhibit a more intense inflammatory process, both locally and systemically[13, 14]. Furthermore, there are multiple published results suggesting that these chronic bronchitic individuals are more likely to exhibit bronchial colonization[13, 15], which has been linked to an increased pro-inflammatory response, including neutrophil elastase[16-19]. Unfortunately, all of these studies have utilized limited, culture-based techniques. **No study using state of the art molecular techniques has been conducted with bronchoscopic samples from well characterized COPD patients, including those that experience cough and sputum production**. Similarly, previous reports using culture-based techniques have suggested that individuals with a history of recurrent exacerbations exhibit greater local inflammatory change and bacterial colonization. **This substudy will allow an examination of the change in microbiota in well-characterized COPD patients over 16 weeks.**

Additionally, in prior studies, we have shown that increases in epithelial mucin stores are associated with increased airway obstruction in COPD [20]. These findings parallel the increase in MUC5AC gene expression observed in airway epithelial brushings from the same subject. This airway mucus phenotype may be associated with the classical clinical phenotype of chronic bronchitis, with bacterial colonization and with frequent exacerbations of COPD. **Therefore, using airway epithelial brushing from this study, we will measure gene expression levels of airway epithelial mucins (using PCR)**. There is extensive data linking neutrophil elastase with

mucin production[21-23]. In addition, *Pseudomonas aeruginosa* induces mucin production in epithelial cells via epidermal growth factor receptor [24]. **This substudy will allow a careful assessment of change in airway microbiota, airway inflammation and mucin production with therapy from a selective, potent PDE4 inhibitor.**

Data from *in vivo* animal models suggest a potential role for PDE4 inhibitors in COPD. Endotoxin can lead to the recruitment of neutrophils to the airway which is also seen in COPD. Neutrophil recruitment to the airways was inhibited by approximately 50% mice deficient in both PDE4B and PDE4D; even greater inhibition was observed in rolipram-treated wild-type mice[25]. Smoking-induced recruitment of neutrophils to the airways was attenuated by roflumilast. Treatment-induced changes in lung inflammation were associated with short-term decreases in neutrophilia in BAL fluid and long-term by reduced lung macrophage density on histology26. A provocative study examined the effect of roflumilast exposure on cigarette smoke induced acute lung inflammation and chronic lung damage in mice[26]. Acute smoke exposure caused a 5-fold increase in BAL fluid neutrophils that was decreased by 30% with roflumilast treatment. Chronic exposure caused a 1.8-fold increase in macrophage density and structural emphysema; roflumilast at higher dose (5 mg/kg vs 1 mg/kg) decreased macrophage density by 70% and fully prevented other changes. Subsequent analyses documented that chronic smoke exposure increased neutrophil volume density, macrophage, dendritic cell, Blymphocyte, CD4+, and CD8+; this was attenuated by the higher dose of roflumilast[27]. Importantly, roflumilast attenuates MUC5AC gene and protein expression induced in epithelial cells by epidermal growth factor (EGFR) [28]. In a recent study of an inhaled EGFR antagonist, the degree of EGFR inhibition achieved at the highest doses of antagonist correlated with reduction in airway epithelial mucin stores [29] linking the EGFR pathway with mucin production in COPD. Interestingly, biofilm production, a crucial component of *pseudomonas aeruginosa* pathogenicity, is modulated by enzymatic pathways with phosphodiesterase activity[30]. **It is biological plausible that phosphodiesterase inhibition may directly impact the microbiome or the impact of these organisms on host response.**

Proof of concept documenting an anti-inflammatory effect of a PDE4 inhibitor, cilomilast, has been published in a 12 week study; bronchial biopsies demonstrated reduction in CD8+ and CD68+ cells [31]. *Post-hoc* analysis of two one year studies of roflumilast demonstrated that the greatest benefit was seen in subjects who were classified by the investigator as experiencing chronic bronchitis, more severe airflow obstruction and increased amounts of cough and sputum; this finding was particularly relevant for the decrease in AECOPD rate [32]. Whether this represents a distinct phenotype of COPD patients remains unclear 11. Subsequent placebo-controlled studies were performed in COPD patients with an FEV1% predicted < 50%, chronic cough and sputum production, and at least one documented COPD exacerbation requiring glucocorticoids, treatment in the hospital, or both in the prior year [33] confirmed improvement in prebronchodilator FEV1 and reduction in moderate or severe exacerbations. **It is likely that the beneficial effect of roflumilast represents an attenuation of the inflammatory response to microbial colonization and the associated epithelial mucin generation**. **This substudy will utilize state of the art techniques to longitudinally assess airway microbiota, inflammation and mucin production.**

This substudy hypothesizes that roflumilast exerts a beneficial effect by altering the inflammatory response to the lung microbiota and decreasing mucin. This will be tested in three specific aims: *a) We will examine the longitudinal change in microbiota in roflumilast- and placebo-treated patients over 16 weeks of therapy. Sub aim – We will examine the interaction of mucosal inflammatory response with changes in microbiota. b) We will examine the longitudinal change in epithelial MUC5AC gene expression in roflumilast- and placebo-treated patients over 16 weeks of therapy. Sub aim – We will examine the interaction of mucosal inflammatory response with changes in mucin gene expression.* c) *We will examine the interaction of roflumilast- versus placebotherapy on the changes in microbiota, mucosal inflammation and mucin production.*

Our proposed study leverages an on-going bronchoscopic study which examines the effect of roflumilast on bronchial inflammatory cell infiltration. **The additional data collection focusing on change in the airway microbiome and epithelial mucin production will provide unique insight into the effects of a potent antiinflammatory therapy in COPD.** Importantly, this goal will be achieved at minimal cost and risk or burden to participating subjects and investigators.

2) BRONCHOSCOPIC PROCEDURE

Recent data suggest that some individuals exhibit a microbiota that is similar in the mouth and bronchoscopic samples. This has raised questions regarding oral contamination. Similarly, some have suggested that fiberoptic bronchoscopes may contain microbial DNA despite sterilization. As such, we have slightly modified the bronchoscopic protocol to minimize these influences. These modifications include: a) Prior to each bronchoscopic procedure the study subject will rinse their mouth with 20 cc of saline for 20 seconds. They will spit this solution into a container which will be labelled with study subject number and the date of the procedure. These samples will then be immediately frozen at -80 degrees C, stored and batch-shipped to the University of Michigan. b) Prior to each bronchoscopic procedure the bronchoscopist will suction 10 cc of sterile saline from a container through the suction channel of the bronchoscope and collect this sample in a separate container. These samples will be labelled with study subject number and the date of the procedure. These samples will then be immediately frozen at -80 degrees C, stored and batch-shipped to the University of Michigan.

3) BRUSHING PROCEDURE

Bronchial Biopsies are already being performed in this study for the primary outcome in all patients (difference in number of inflammatory cells CD8+ in bronchial biopsy tissue specimen from randomization visit V2 to the end of the intervention period (V6). In selected Subjects three protected brush specimens will be collected during the bronchoscopy procedures at visits V2 and V6. If performed, these specimens should be collected after the bronchial biopsy procedure and always at the same part of the lobe bronchus as specified below. An additional Subject Informed Consent will be collected prior to participation of any Subjects in this subtrial.

A. Two protected brush specimens will be performed in the right middle lobe bronchus for microbiome studies. The BARD Disposable Microbiology Brush will be introduced into the segment and the plug “dropped”. Advance the brush and gently brush the bronchial mucosa (about 12.7 mm) while rotating the brush 360 degrees. After about 7 seconds of brushing, retract the

brush completely into the inner catheter. Then retract the inner catheter into the outer catheter by pulling the blue and white section apart. Withdraw the microbiology brush assembly from the bronchoscope. See description below on how to remove the brush. Collect one additional microbiological brush sample *with the second provided protected brush*. B. One cytological brushing will then be performed in the right middle lobe bronchus for gene expression studies. The cytological brush (ConMed, Catalog # 149, 3mm diameter x 11mm long, phone # (800) 448-6506) will be used to gently brush the bronchial mucosa (about 12.7 mm) while rotating the brush 360 degrees.

4) PROCESSING OF THE BRUSHING MATERIAL ON SITE

A. Microbiome Brush handling: (n=2)

1) Remove each brush sample by first wiping the outer catheter approximately 5 mm distal to the inner catheter with an alcohol prep (proposed cut site). Then cut the outer catheter at the alcohol cleansed site, and discard.

2) Then, completely advance the inner catheter. Wipe the inner catheter about 5 mm distal to the brush tip (proposed cut site) with another alcohol prep, and then cut the inner catheter at the alcohol cleansed site.

3) Advance the brush directly and completely into a sterile dry 2 ml eppendorf tube and kept on dry ice.

4) Repeat with new tube for each brush.

5) Samples will then be immediately frozen at -80 degrees C, stored and batch-shipped to the University of Michigan.

B. Cytological Brush handling: (n=1)

1. Remove the brush by advancing the brush tip into a 2 mL screw cap tube containing 1mL RNase-Free PBS on ice. Cut the wire using sterile scissors and close the cap over the brush.

2. Store brushing tube on ice until further processing.

3. After the bronchoscopy, vortex this tube at setting 3-4 (low speed such that only a small amount of foam results) for ~ 3 minutes.

4. Remove brush.

5. Centrifuge tube at 300 x g for 5 minutes

6. Discard supernatant

7. Disrupt the cells by adding 600 µl QIAzol Lysis Reagent. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube.

8. Vortex for 1 minute on highest setting to lyse cells. Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

9. Transfer suspension to 1.5 mL Eppendorf tube (with O-ring)

10. Samples can then be stored at -80 degrees C until they are batch shipped to the University of Michigan. Batch shipping will occur after sponsors request in a frozen state from clinical site via PPD's Global Central Labs to the following address:

A) From clinical site to PPD Global Central Lab

PPD´s Global Central Labs

Phone
Fax

B) From PPD`s Global Central Lab to University of Michigan

University of Michigan Medical Center

5) PROCESSING THE SAMPLES

A. Microbiome sample processing

1. Sample Storage and Processing:

Brushes will be received in Michigan and transferred to a sterile bead beating tube. The eppendorf tube used for transport will be washed with 500 µl of sterile bacterial lysis buffer (BLB (Roche)) and the wash transferred to the bead beating tube containing the brush. At this point the bead beating tube can be frozen at -80°C or DNA will be immediately extracted.

2. DNA Extraction

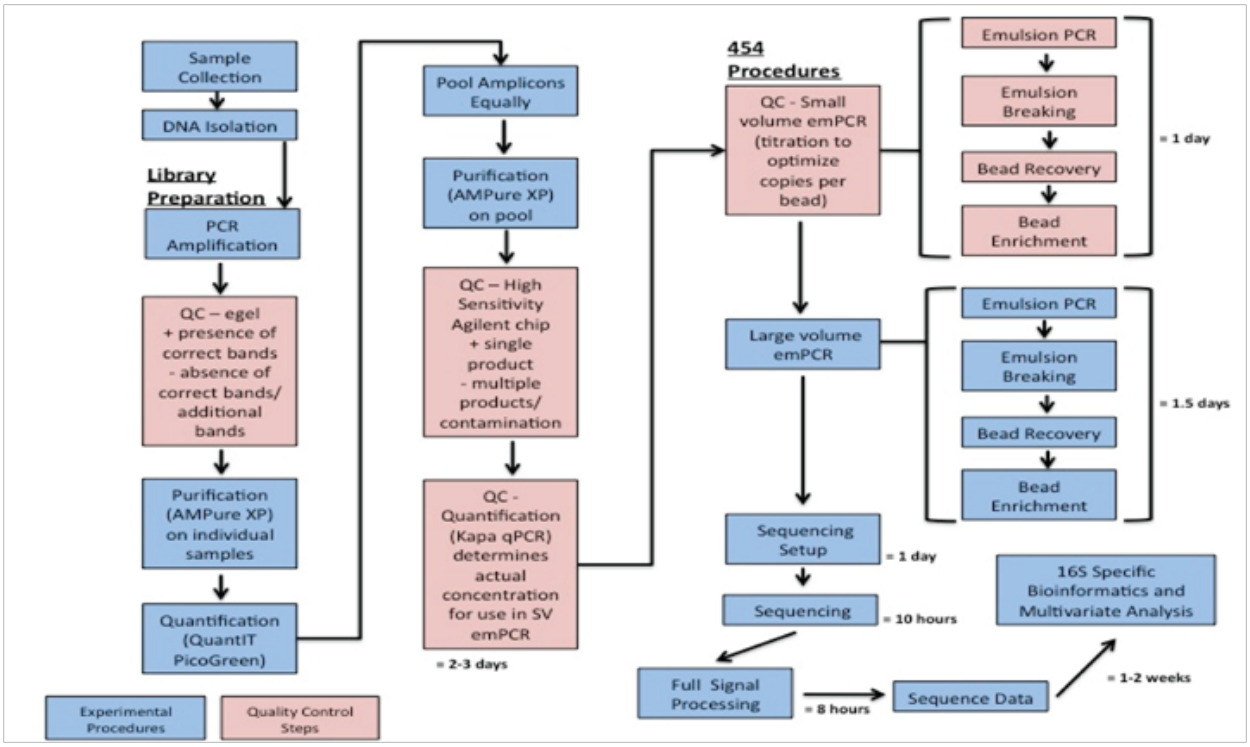
Bead beating tubes containing the sample brush and BLB will be will be homogenized in a bead beater for 2 minutes followed by incubation with Proteinase K (Qiagen) at 65°C for 10 minutes. Next, another 2 minute bead-beating step followed by inactivation of the enzyme with a 10 minute incubation at 95°C. The DNA or RNA will then be purified from the lysate using the MagNA Pure method of nucleic acid purification by magnetic beads (Roche). We have previously found this method to be very effective in the isolation of microbial DNA from cytology brushes. However, should this method prove inadequate we will switch to the PowerSoil method (MolBio) that has had good success in the Human Microbiome Project (HMP). DNA will be quantified using a Nanodrop 2000.

3. 454-Pyrosequencing of Bacterial Metagenomes

454-Pyrosequencing is a method by which hundreds of thousands of long-read sequences (~450-500 bp) can be generated simultaneously from a sample. This technique is therefore perfect for metagenomic studies of amplicon libraries. Both a GS-FLX using the Titanium chemistry and a

GS-Junior will be available for pyrosequencing and will be utilized depending on sample size and the number of reads per sample desired. The flow diagram below (**Figure 3**) details the process of generating metagenomic data from DNA samples. The first step is the isolation of total DNA from the sample. Once DNA is obtained, amplicon libraries will be generated by selectively amplifying the V5-V3 region using PCR primers that have been specifically designed to work with the 454 pyrosequencer. The forward primer has also been modified to include one of 96 different 6 bp barcodes which allow for multiple samples to be run simultaneously and bioinformatically sorted later. This latter modification to the standard 454 protocols significantly decreases the persample cost while increasing the sample throughput.

Figure 3: Workflow of Metagenomic Sequencing



Once the amplicon library is generated, it undergoes quality control to ensure that only a single product will be submitted to the sequencing process and that the product is the product of interest. From this point through the end of sequencing, Roche protocols will be followed exactly. Once the sequencing is completed, image analysis and signal processing software will be used to produce fasta-formatted nucleotide sequence data. This data will then be analyzed to characterize the metagenome of the samples.

B. Endobronchial brush sample processing

RNA will be prepared from the QIAzol reagent according to manufacturer instructions. RNA quality will be assessed using by capillary electrophoresis (Agilent Bioanalyzer) and concentration

will be measured using a UV spectrophotometer (Nanodrop, Thermo Scientific). For analysis of mucin gene expression (MUC5AC, MUC2, MUC5B), two-step qPCR will be performed as described previously 20. cDNA synthesis will be carried out by using 20 ng of total RNA and BD Clontech (Mountain View, CA) Powerscript Reverse Transcriptase with random hexamers for priming. Then, multiplex preamplification will be performed by using one-fifth of the resultant cDNA, Advantage 2 Polymerase (BD Clontech), and 5 pmol of each outflanking primer. Multiplex hot-start amplification will be done for 5, 10, 15, and 20 cycles to ensure that the reaction remains in the exponential phase of PCR and the substrates are not limiting. Real-time PCR gene quantification will then be performed on the amplified cDNA by using TaqMan probes (Applied Biosystems, Foster City, CA) and Universal Master Mix (Invitrogen, Carlsbad, CA). Transcript quantification will be run on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). Cycle threshold values obtained for each gene will then be converted into relative transcript copy numbers based on logarithmic transformation and linear regression of prior data, as described previously in [34]. Transcript copy numbers will be normalized by using a two-step approach. First, the amount of amplification product used in TaqMan profiling will be normalized on the basis of housekeeping gene expression. Then a panel of four housekeeping genes will be measured during TaqMan profiling (GAPDH, ubiquitin, EEF1A1, and PPIA), and the geometric mean value of the two housekeeping genes most stably expressed across the samples will be used for normalization[35].

5. REPORTING

A. Metagenomic Data Analysis

Analyzing metagenomes using 16S data falls broadly into two categories: Classification-based (database directed) or OTU-based (OUT - Operational Taxonomic Unit). Of database directed approaches, BLAST is the most common algorithm used for non-16S samples; however, for differentiation of 16S samples BLAST is not optimal because the algorithm cannot take into account the secondarystructural elements that make 16S unique (and help to differentiate organisms). To solve this problem we utilize RDP-classifier a naïve-Bayesian classifier, which is fast and secondary-structure aware. Using this method we can accurately identify the bacterial organisms present. To prepare the data for analysis, the data will first be bioinformatically sorted so that sequence and identifier sit on the same line. Next, sequences less than 200 bp in length will be filtered out. Finally, the barcode incorporated into the sequencing primer will be used to sort samples. Sequences that either lack a barcode or possess a non-matching barcode will be removed from analysis. The resulting file will then be run through RDP-Classifier run locally so as to increase the speed of analysis. Once completed the classified data is imported into R and a community table detailing the bacterial membership and abundance of each sample will be generated using several in-house designed scripts.

The limitation to any database-directed approach is the inability to identify an organism that does not exist in the database. This is where OTU-based methods excel. OTU-based methodologies cluster sequences based percent sequence identity so no database is needed. Previous studies have demonstrated that 3% difference across the whole 16S, or the V3-V5 region, corresponds to species-level taxonomic differentiation. Thus, by using an OTU-based it is possible to characterize

the bacterial population in greater detail than traditional classification schemes allow. Mothur, an open source application explicitly designed for the analysis of large pyrosequencing data sets (http://www.mothur.org), is the software that we employ for OTU-based analysis.

Analysis of pyrosequencing data using Mothur begins with reading the raw data and breaking it up according to the barcodes that define the samples, and setting the sequences to similar lengths. Next, the sequences are de-replicated which creates a much smaller pool of unique sequences to proceed forward in the analysis. To adjust for PCR error, sequences that contain single base-pair differences are grouped together using a pseudo single-linkage clustering algorithm. Sequences are then aligned, using a fast Needleman aligner, to the Silva 16S database (www.arbsilva. de). This database has been empirically been shown to produce the most accurate 16S alignments. Once aligned, the chimeric sequences are removed and a distance matrix is generated which relates each sequence to every other sequences based on sequence identity. The final step in the generation of OTUs is the grouping together of sequences together that differ by a specified amount (e.g. 5% difference). These OTUs can also be classified to give a taxonomic identity. Because all of the above-mentioned steps are extremely computationally intensive processes, Mothur is parallelized and can be run on a computer cluster as needed.

The next stage in the characterization of the bacterial community is to employ some of the tools for multivariate analysis that are frequently used in the field of ecology. Diversity statistics are commonly used to assess the diversity, based on both the membership (what organisms are present) and the structure (how much of each organism are present), estimate overall richness of the community, and compare communities against one another. These complex communities can be visualized using ordination methods such as Non-metric Multi-dimensional Scaling (NMDS) and Principle Components Analysis (PCA) which enable us to assess community relatedness based on how samples cluster and determine which members of the community have the greatest influence on the clustering. Models are then constructed to examine the significant drivers of difference between groups and test the significance of available metadata (characteristics of inflammatory infiltrate, cytokine expression patterns, etc.). A detailed Statistical analysis plan will be prepared and finalized prior unblinding of the main study.

B. Mucin gene expression analyses

For mucin gene expression data, normalized transcript copy number will be logtransformed so that it acquires a normal distribution. Changes in the expression of each airway epithelial mucin (MUC5AC, MUC2 and MUC5B) will be assessed separately, and change in MUC5AC will be considered a priori the secreted mucin of primary interest as it is the predominant mucin in airway epithelial cells. Increases in mucin stores parallel the increase in MUC5AC gene expression observed in endobronchial brushings. A detailed Statistical analysis plan will be prepared and finalized prior unblinding of the main study.

C. Reporting of the data

The results of these sub-study analyses will be reported in a separate Clinical Trial Report independently to the main study results.

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Appendix J For Germany: The TRANSCRIPTOM Sub study only valid for the site of

Title of the local, site specific substudy:

Gene expression profiling of inflammatory sputum cells in COPD – an exploratory research approach to study the molecular anti-inflammatory treatment effects of Roflumilast in study RO-2455-402-RD

Contents

1.1 Responsible site and investigators 144
1.2 Introduction.....144
1.3 Study conduct.....144
1.4 Platform for transcriptom analysis.....145
1.5 Statistical analysis.....145
1.6 Reporting.....145
1.7 Reference List 145

1.1 Responsible site and investigators

Professor of Medicine Dept of Internal Medicine Christian Albrechts University Kiel, Germany Head, Dept of Pulmonary Medicine and Medical Director

Tel.: Fax.: E-Mail:
Internet:

and

Pulmonary Research Institute

T
F
E-Mail:
Internet:

1.2 Introduction

Chronic inflammation in COPD is a complex interplay of various cellular and molecular mechanisms resulting in airway neutrophilia, which can noninvasively be assessed by investigation of induced sputum samples. Inhibition of the Phosphodiesterase 4 (PDE4) by Roflumilast has been demonstrated to reduce airway neutrophilia and associated biomarkers in COPD before. We propose to broaden the understanding of the anti-inflamamtory effects of Roflumilast by comprehensive gene expression profiling of induced sputum samples before and after treatment with Roflumilast.

The variables of primary interest in sputum (differential cell count and biomarkers in sputum supernatant) are not affected. The chance to explore broad anti-inflamamtory effects of Roflumilast on the molecular level after three months of treatment outweighs the risk to observe no changes in favor of Roflumilast

This Transcriptom Sub study will be only conducted at the site of , Grosshansdorf, Germany.

1.3 Study conduct

The site Grosshansdorf plans to include at least 16 patients or more in the study RO-2455-402-RD.

Sputum samples collected during the clinical visits at the site Grosshansdorf will be processed according to the sputum manual of study RO-2455-402-RD. Differential cell count and biomarker analyses will be performed as described in the protocol. The remaining sputum cell pellet (a leftover after sputum processing) will be fixed with HOPE and embedded in paraffin. The site

recently validated this novel technique using inflammatory cells from bronchoalveolar lavage, which were subjected to standard molecular techniques such as gene expression profiling, quantitative polymerase chain reaction, in situ hybridization, and immunohistochemistry [1]. In preparation of the study RO-2455-402-RD the investigators at the site recently finished the validation of the biomarker analysis panel of Rules-Based Medicine for sputum supernatant (collaboration Grosshansdorf and Takeda) and adopted the method of HOPE-fixed BAL cells for sputum [2]. Comparing the transcripts of sputum cells from COPD patients with the transcripts of healthy controls the investigators of the site found a total of 8.922 genes to be differentially expressed in COPD (log 2 fold change >2.1 up- or down-regulated). Genes that were differentially expressed with a log 2 fold change >4 encompassed genes coding for neutrophil chemoattraction and impaired phagocytosis, both which are fundamental mechanisms underlying chronic inflammation in COPD.

1.4 Platform for transcriptom analysis

Agilent technology (Agilent 4 × 4 4k whole human genome microarrays scanned with the Agilent scanner, Agilent Technologies, Santa Clara, CA).

1.5 Statistical analysis

The statistical analysis will described in a separate statistical analysis plan.

1.6 Reporting

The data will be analysed for explorative reasons. The analyses will be described in a separate statistical analysis plan and finally the data in a separate report. It will be not part of the main SAP or main CSR.

1.7 Reference List

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Appendix K For UK: The Airway Reflux Sub study only valid for the site of

Title of the local, site specific substudy:

The Airway Reflux sub study of the Roflumilast 'biopsy study' RO-2455-402-RD. An exploratory research approach to study the changes of the degree of airway reflux

Contents

1.1 Responsible site 147
1.2 Introduction.....147
1.3 Study conduct.....147
1.4 Study assessments.....148
1.5 Statistical analysis.....148
1.6 Reporting.....148
1.7 Restech product specification and information.....149
1.8 Hull Airways Reflux Questionnaire (HARQ).....151
1.9 Reference List 152

1.1 Responsible site

Head of Division of Cardiovascular and Respiratory Studies
University of Hull
Castle Hill Hospital

Tel:
Fax:
Email:

1.2 Introduction

Roflumilast is an orally active agent with demonstrable clinical activity in preventing exacerbations of COPD. It has been shown to have anti inflammatory activities within the airways. The parent study RO-2455-402-RD to which this protocol applies is designed to understand the molecular mechanisms of the reduction of inflammation within the airways of patients. How this reduction in inflammation occurs is at present unknown.

It is becoming increasingly apparent that a number of exacerbations of COPD are due to episodes of microaspiration into the airways. Evidence for this obtained from epidemiological surveys showing that patients with exacerbations have a high incidence of gastroesophageal reflux disease. Many patients however do not exhibit the full symptom profile of GERD. The site has recently demonstrated that the majority of such patients have a symptom complex consistent with non acid reflux and microaspiration leading to bronchitis. The most effective agent currently in use for preventing exacerbations of COPD is azithromycin[1]. The site has suggested that this efficacy resides the well established effect of azithromycin on GI motility, preventing such episodes of microaspiration[2]. In this protocol, patients entering the RO-2455-402-RD -biopsy study will undergo additional non invasive assessments to determine their degree of airway reflux and ascertain whether roflumilast therapy has any beneficial effect on the frequency of these episodes.

This Airway Reflux Sub study will be only conducted at the site of , East Yorkshire, UK.

1.3 Study conduct

Patients will be admitted to the RO-2455-402-RD biopsy trial as per the agreed criteria and protocol. Those patients under the care of the Clinical Trials Unit at Castle Hill Hospital will be asked to volunteer to take part in the sub study. All patients have to give their consent to participate at this sub study. The investigators is anticipating that approximately 12 patients or more will be recruited. Subjects will be assessed for evidence of airway reflux using two techniques.

1. Patients will be administered the Hull Airways Reflux Questionnaire (HARQ). Briefly, this is a validated, self administered, 14 point questionnaire which quantifies the symptom complex associated with airway reflux. In a study of 100 patients with airflow obstruction admitted to our hospital Trust over 80% scored above the upper limit of normal (13 out of 70) indicating positive symptomatic evidence of airway reflux in the etiology of exacerbation. The HARQ has been demonstrated to be responsive to therapy and have a high degree of reproducibility and internal validity[3].
2. The Restech pharyngeal pH probe will be used to establish 24 hour levels of gaseous airway reflux. The monitoring device has been used in over 200 respiratory patients at Castle Hill Hospital. The test is non-invasive and since the small probe is placed in the posterior pharynx, comfortable for use, even in those with advance respiratory disease. Ambulatory patients will be studied in their home environment for 24 hours at baseline and on therapy. The Ryan Score will be used to determine the degree of airway reflux present in each patient.

1.4 Study assessments

Patients will undertake the HARQ questionnaire and Restech analysis at baseline or have had these assessments made in the three months preceding entry into the study. Tests will be repeated at the end of treatment period prior to their final biopsy. The RO-2455-402-RD -biopsy study has a 6 week run in where there is a treatment change for patients ie if on steroids these will be withdrawn. The biopsy sample will therefore be taken at V2 (wk6) of run-in. Thus, if not undertaken earlier, the Restec and HARQ assessments will be performed 24 hours before V1 (2 weeks prior to Randomisation visit 2). The final Restech analysis, on Roflumilast /Placebo, will be performed 24 hours prior to V5, following 14 weeks of Roflumilast. There is a visit 6 at 16 weeks treatment but the biopsy is to be performed at this visit so measurements should be performed prior to the time point.

1.5 Statistical analysis

Comparison of baseline HARQ score to end of treatment will be compared by non-parametric statistics (Wilcoxon's rank-sum test). Restech analysis will use as a primary end point change in Ryan score, with, as a secondary end point, the number of episodes of fall in pharyngeal pH.

1.6 Reporting

The data will be analysed for explorative reasons. The analyses will be described in a separate statistical analysis plan and finally the data in a separate report. It will be not part of the main SAP or main CSR.

1.7 Restech product specification and information

product SPECIFICATIONS

Dx-pH Probe™

MODEL	CHANNELS	SHAFT DIAMETER	TIP DESCRIPTION
Dx-201	1	4.6 Fr/1.5mm	teardrop, red LED

Hypoallergenic--Contains no latex or PVC.

Dx-Transmitter™

MODEL	DIMENSIONS	WEIGHT	BATTERY	COMPLIANCE
Dx-300	*l* 2.75" *w* 0.88" *h* 0.60"	25 g	Coin cell CR 1632	IEC 60601-1 FCC 15.247

Dx-Recorder™

MODEL	DIMENSIONS	WEIGHT	BATTERY	DATA STORAGE
Dx-500	*l* 4.50" *w* 2.75" *h* 1.25"	150 g	2 AA Alkaline	SD™ Memory Card

Dx-Calibration Vials™

MODEL	CONTENTS
Dx-020	pH 7.0 Calibration Solution pH 4.0 Calibration Solution } NIST Standards Rinse Solution

Dx-Sleep Adapter™ (can custom fit to most specifications)

MODEL	CABLE	OUTPUT	OUTPUT VOLTAGE
Dx-400	60"	(+1V) 1/8" Mono male audio jack	(+1V) 0 to 1 VDC

Dx-pH DataView Lite™

FEATURES
PC based, graphical interface software that sets up study, downloads completed study, edits and relates data, prints graphs and reports, stores patient files

The Dx-pH Measurement System (shown above) monitors pH in the airway, assisting the physician in determining the relationship between Laryngopharyngeal Reflux and various conditions including:

- *Recurrent Laryngitis*
- *Chronic Cough*
- *Sinusitis*
- *Asthma*
- *Sleep Disordered Breathing*
- *Chronic Obstructive Pulmonary Disease*

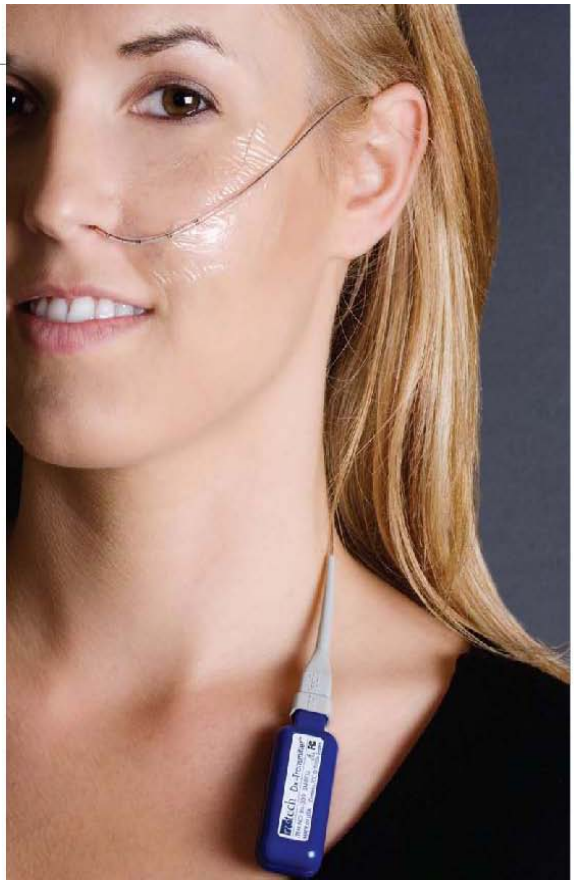
about RESTECH

Restech is a leader in engineering world-class medical technologies that provide comfortable, reliable solutions to assist physicians in the diagnosis of respiratory and reflux related health problems. The staff and engineers at Restech are committed to excellent customer service and support as well as a continued pursuit of innovative instrumentation for your practice.

Respiratory Technology Corporation
10804 Willow Ct. Ste. B
San Diego, CA 92127

web www.restech-corp.com
email contact@restech-corp.com
tel 800.352.1512 / 858.673.3700
fax 858.673.3783

CAUTION: Federal Law (USA) restricts these devices to sale by or on the order of a physician.



restech

shed a new light on airway pH

airway pH: WHY MEASURE

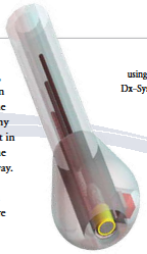
Until the introduction of Restech's Dx–pH Measurement System, accurate, real-time measurement of airway pH was not possible. Physicians relied largely on subjective and empiric drug trials to confirm a diagnosis.

Acidic or alkaline extremes cause damaging effects in the airway and even worse damage to the lungs. The longer and more severe the exposure, the greater the corresponding damage. Complicating the effects of extreme pH is the similarity in symptoms and visual appearance of the epithelium. Likewise, allergies, vocal abuse, sleep apnea and laryngopharyngeal reflux can manifest as symptoms that are difficult, if not impossible, to differentiate.

restech's SOLUTION

The Dx–pH Measurement System measures and records airway pH every ½ second for up to 48 hours, while the patient inputs clinically relevant information such as meals, symptoms, and supine position with the press of a button. The patented, miniaturized antimony sensor and reference electrode are housed 0.002" apart in the tip of the Dx–pH Probe, enabling it to measure the aerosolized particles of refluxate in your patient's airway.

All pH data is transmitted wirelessly and stored on an SD card for review with the Dx–pH DataView software program.



Dryout Detection
using hydration monitoring circuitry, the Dx–System records pH 15 if dryout occurs

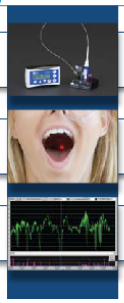
Teardrop Shape
minimizes fouling

Red Light-Emitting Diode (LED)
assists in visual placement

Greater Sensitivity
with its Antimony-E design and sensor face size < 1mm

Downward Aim
reduces masking

how IT WORKS



1 SET UP STUDY
Follow the Dx–Recorder prompts to complete the simple process (usually set-up by office staff).

2 PLACE THE PROBE
Insert the catheter through the naris and adjust the position until the flashing LED in the probe tip appears behind the soft palate.

3 DOWNLOAD DATA
The intuitive DataView software calculates reflux events, categorizes correlations and provides a graphic representation of airway pH levels.

the RESULTS

An 18-48 hour picture of your patient's airway pH levels provides useful evidence-based data to assist with diagnosis and appropriate treatment. Choosing an effective course of treatment just got easier.

Your patient will present with a test that is:

- *Alkaline*
- *Mixed Alkaline and Acidic*
- *Acidic*
- *Normal pH (Negative)*

The pH data and patient input information captured during the study (meal periods, symptom occurrence and supine period) are plotted on the data graph so you can easily develop an appropriate treatment pathway.

The Dx–System can also be used to monitor airway pH levels during sleep studies with a direct plug-in to a PSG machine. Monitoring airway pH during a sleep study is particularly relevant for correlating silent reflux with respiratory symptoms and arousals from sleep.

Why Choose the Dx–pH Measurement System?

- Accurate airway pH measurement
- Faster diagnosis of LPR
- Greater precision in treatment pathway design
- Avoidance of empiric trial and unnecessary exposure to medication
- Up to 48 hours of detailed and charted information
- Time capture & symptom correlation
- Ability to study nocturnal supine period

in YOUR PRACTICE

Get the information you need. Give your patients the care they deserve.

Integrate the Dx–pH Measurement System into your practice today. Restech provides on-site installation and training, followed by 24/7 technical support to help you and your staff gain comfort and confidence with this technology–all at no additional cost.

Call 800.352.1512 or *visit* www.restech-corp.com.

1.8 Hull Airways Reflux Questionnaire (HARQ)

REFLUX COUGH QUESTIONNAIRE

Name: _____

D.O.B: _____ UN: _____

DATE OF TEST: _____

Please circle the most appropriate response for each question

Within the last MONTH, how did the following problems affect you? **0 = no problem and 5 = severe/frequent problem**						
Hoarseness or a problem with your voice	0	1	2	3	4	5
Clearing your throat	0	1	2	3	4	5
The feeling of something dripping down the back of your nose or throat	0	1	2	3	4	5
Retching or vomiting when you cough	0	1	2	3	4	5
Cough on first lying down or bending over	0	1	2	3	4	5
Chest tightness or wheeze when coughing	0	1	2	3	4	5
Heartburn, indigestion, stomach acid coming up (or do you take medications for this, if yes score 5)	0	1	2	3	4	5
A tickle in your throat, or a lump in your throat	0	1	2	3	4	5
Cough with eating (during or soon after meals)	0	1	2	3	4	5
Cough with certain foods	0	1	2	3	4	5
Cough when you get out of bed in the morning	0	1	2	3	4	5
Cough brought on by singing or speaking (for example, on the telephone)	0	1	2	3	4	5
Coughing more when awake rather than asleep	0	1	2	3	4	5
A strange taste in your mouth	0	1	2	3	4	5

TOTAL SCORE_____ /70

Version 4 December 07

1.9 Reference List

1. Albert,R.K. *et al.* Azithromycin for Prevention of Exacerbations of COPD. *N Engl J Med* **365**, 689-698 (2011).
2. Crooks,M., Hart,S.P. & Morice,A.H. Azithromycin in COPD. New England Journal of Medicine. 2011.
3. Morice,A.H., Faruqi,S., Wright,C.E., Thompson,R. & Bland,J.M. Cough hypersensitivity syndrome: a distinct clinical entity. *Lung* **189**, 73-79 (2011).

Appendix L Detailed Description of Amendments to Text

This document describes changes in reference to Protocol Incorporating Amendment No. 12

Page 3, 1.1 Contacts

Existing Text

Medical Monitor:

ASS MED DIR PHARMACOVIGILANCE
UK

New Text

Medical Monitor:

Associate Medical Director, Pharmacovigilance
PPD Bulgaria EOOD

Hotline:
SAE Fax:

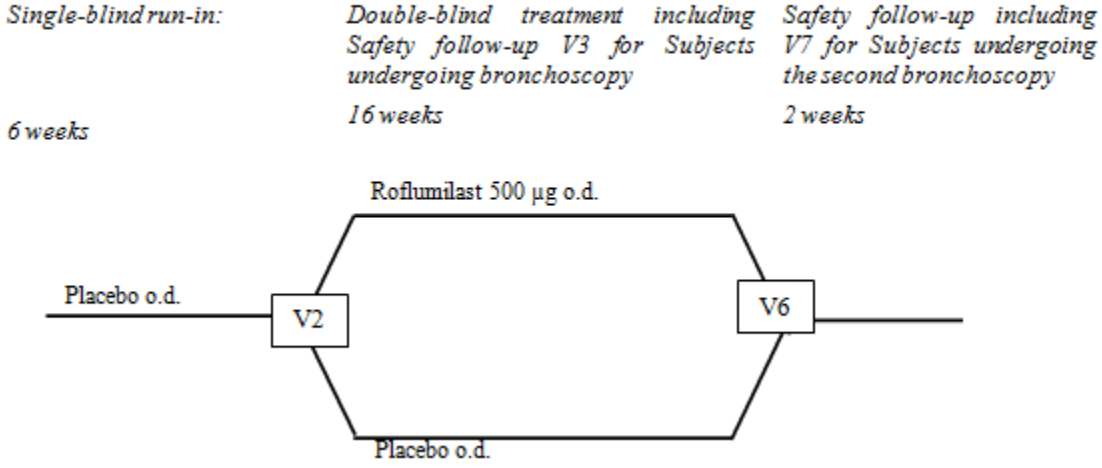
Rationale for Amendment

To reflect change in Medical Monitor.

Page 16, Section 6.1 Study Design

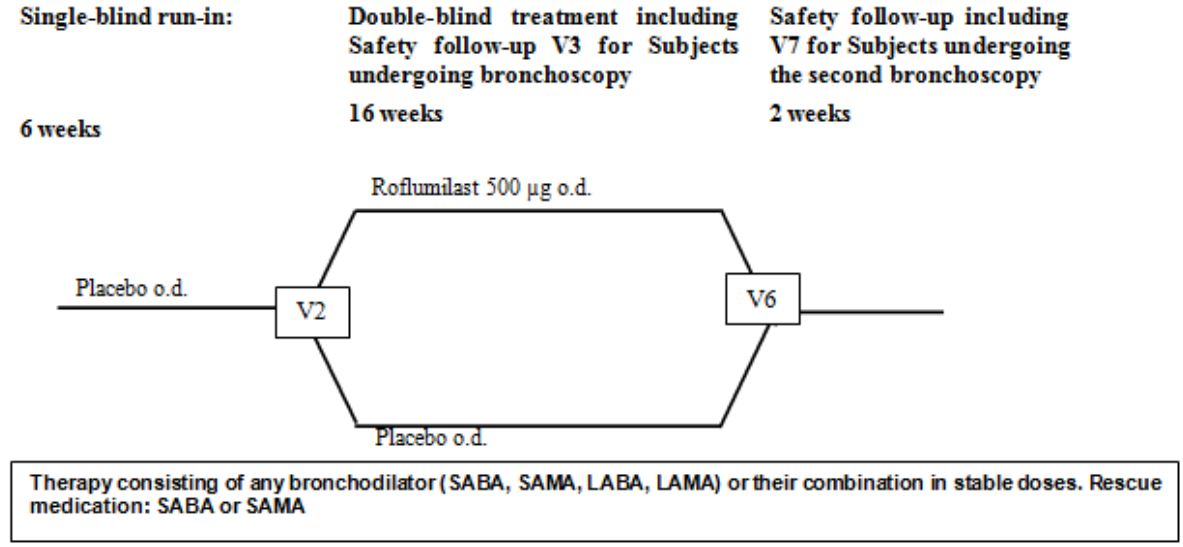
Existing Text

Figure 6.a Schematic of Study Design



Revised Text

Figure 6.a Schematic of Study Design



Therapy consisting of any bronchodilator (SABA, SAMA, LABA, LAMA) or their combination in stable doses. Rescue medication: SABA or SAMA

Rationale for Amendment

Wording around the use of bronchodilators over the course of subjects study participation which was in earlier versions of the protocol was inadvertently omitted and is therefore being reintroduced for clarity.

Page 27 Section 6.2 Justification for Study Design, Dose and Endpoints

Existing text

Bronchoscopy is a relatively safe procedure. Serious risks from bronchoscopy, such as an air leak or serious bleeding are reported in less than 5 %. Other risks associated with the procedure are as follows: bleeding, discomfort and coughing, reduced oxygen, lung leak or collapse (pneumothorax) [12]. Given that the safety of the volunteer Subject is of paramount importance, bronchoscopy has an invaluable place in airway research. The conduct of the clinical trials should be based on well established principles of trial design [13]. The individual risk of the Subjects in this trial in regard to the conducted method bronchoscopy/bronchial biopsy is seen as minimized based on several embedded protocol aspects. The bronchoscopy and bronchial biopsy will only be conducted at experienced investigational sites which have shown in previous clinical trials to be capable to perform bronchoscopies in a standardized and safe manner. Same predefined, standardized procedures will be followed, the sites will be individually trained on the trial related procedures, and the quality of the biopsied material will be validated for each site. Dedicated safety follow-up visits have been implemented in the trial schedule subsequent to each visit with a bronchoscopy assessment. In addition, the sputum collection sampling has been separated from the bronchoscopy visits in order to not perform too many interventions on the same day.

Revised text

Bronchoscopy is a relatively safe procedure. Serious risks from bronchoscopy, such as an air leak or serious bleeding are reported in less than 5 % of procedures. Other risks associated with the procedure are as follows: bleeding, discomfort and coughing, reduced oxygen, lung leak or collapse (pneumothorax) [12]. Bronchoscopy has an invaluable place in airway research. Given that the safety of the volunteer Subject is of paramount importance, the conduct of the clinical trials should be based on well established methods [13]. The individual risk to the Subjects from the bronchoscopy/bronchial biopsy procedure is as minimized based on several principles implemented within the protocol. The bronchoscopy and bronchial biopsy will only be conducted at experienced investigational sites which have shown in previous clinical trials to be capable of performing bronchoscopies in a standardized and safe manner. The same predefined, standardized procedures will be followed, the sites will be individually trained on the trial related procedures, and the quality of the biopsied material will be validated for each site. Dedicated safety follow-up visits have

been implemented in the trial schedule subsequent to each visit with a bronchoscopy assessment. In addition, the sputum collection sampling has been separated from the bronchoscopy visits in order to not perform too many interventions on the same day.

Rationale for Amendment

Fix minor gramatiacal errors.

Page 34, Section 7.5 Diet, Activity Control

Existing Text

Subjects will be instructed to fast overnight for at least 8 hours prior to returning to the study center for visits where blood samples will be drawn.

Study medication will be administered once daily in the morning after breakfast with water.

Revised Text

Subjects should not take any food or drink overnight for at least 8 hours prior to returning to the study center for all visits. Subjects will also be asked to avoid strenuous exercise for 8 hours prior to each study visit and to avoid smoking for 4 hours prior to each study visit. For visits where subjects will not undergo blood draws or biopsies, the fasting requirement will only be mandated if clinically indicated as per investigator judgment.

Study medication will be administered once daily in the morning after breakfast with water. On study visit days where the subject is required to fast, study medication will be taken with water only.

Rationale for Amendment

Clarify the requirement for subjects to avoid strenuous exercise as well as smoking prior to visits for all visits. At visits where subjects will not undergo blood draws or biopsies, this requirement will be left to the investigators discretion. Also clarify that despite fasting requirement, a small amount of water is permitted for the purposes of taking medication on visit days.

Page 39, Section 8.1.3 Dose and Regimen

Existing Text

The tablets must be taken orally in the morning after breakfast with some water

Revised Text

The tablets must be taken orally in the morning after breakfast with some water. On study visit days where the subject is required to fast, study medication will be taken with water only.

Rationale for Amendment

Make clear that patients who are fasting in advance of their study visits are permitted to take their tablets with water on visit days.

Page 51, Section 9.1.15.3

Existing Text

9.1.15.3 Brushing Specimens from the Right Lower Lobe for Microbiome Assessments (Sub-Trial in selected Subjects only)

Participation in the Microbiome Sub-Trial is optional, involving only those subjects who voluntarily opt to provide their consent to participate in the Sub-Trial. In these selected Subjects three protected brush specimens will be collected during the bronchoscopy procedures at visits V2 and V6. If performed, these specimens should be collected after the bronchial biopsy procedure in the right middle lobe bronchus.

Revised Text

9.1.15.3 Brushing Specimens from the Right Middle Lobe for Microbiome Assessments (Sub-Trial in selected Subjects only)

Participation in the Microbiome Sub-Trial is optional, involving only those subjects who voluntarily opt to provide their consent to participate in the Sub-Trial. In these selected Subjects three protected brush specimens will be collected during the bronchoscopy procedures at visits V2 and V6. If performed, these specimens should be collected after the bronchial biopsy procedure in the right middle lobe bronchus. In addition, to examine whether microbiota from bronchoscopic samples is from contamination from the fibreoptic bronchoscope or the mouth, a sample of a saline mouthwash and a saline sample drawn

through the suction channel of the bronchoscope will be obtained before each bronchschopic procedure.

Rationale for Amendment

Clarify that bronchial brushing should come from middle lobe of lung and also make clear that a mouthwash sample will also be collected during visits with bronchoscopies.

Page 55, Section 9.3 Schedule of Observations and Procedures

Existing text

The Subject will rest quietly for at least 15 min before any blood for laboratory assessments is drawn (if applicable) and/or any pulmonary function test (see Section 9.1.16) is performed, vital signs are measured, or ECG is performed.

At each visit Subjects should be advised that one tablet of trial treatment once daily should be self-administered in the morning

Revised text

The Subject will rest quietly for at least 15 min before any blood for laboratory assessments is drawn (if applicable) and/or any pulmonary function test (see Section 9.1.16) is performed, vital signs are measured, or ECG is performed. Throughout the course of the trial subjects will need to adhere to the following regulations prior to each trial visit:

- **4h: Avoid smoking**
- **8h: Maintain fasting (refrain from food and drink) and avoid strenuous exercise**

At each visit Subjects should be advised that one tablet of trial treatment once daily should be self-administered in the morning. Subjects will be permitted to take medication with water during this period if this is considered acceptable in the clinical judgement of the investigator.. For visits where subjects will not undergo blood draws or biopsies, the fasting requirement will only be mandated if clinically indicated as per investigator judgement.

Rationale for Amendment

Clarified requirement for subjects to fast and avoid exercise and smoking prior to study visits.

Page 58, Section 9.3.3 Randomization / Visit V2 (6 weeks after baseline visit V0)

Existing text

Further procedures

Subjects will be asked to return to the investigational site for visit V3 ≤2 weeks after visit V2.

***Note**:*

In selected Subjects three protected brush specimens in a lower lobe bronchus will be collected. This is described in Appendix I (see also Section 9.1.15.3)

Revised text

Further procedures

Subjects will be asked to return to the investigational site for visit V3 ≤2 weeks after visit V2.

Rationale for Amendment

Removed text which was both duplicative, and inconsistent for clarity.

Page 72, Section 12.0, Data handling and record keeping

Existing text

All data management activities will be conducted by Sponsor's representative following their Standard Operating Procedures (SOPs)/Working Practice Documents (WPDs). The database will be built by the Sponsor's representative.

SAEs will be reported to and handled by the Sponsor. Queries concerning SAEs will be raised by the Sponsor, and forwarded via Sponsor's representative to the centre on an ongoing basis.

Data storage will be handled by the Sponsor.

Subjects will be identified in the database only by Subject ID, date of birth or age, sex, site, and trial ID. A Data Validation Manual (previously called Data Management Plan) will describe data handling in details. The Data Validation Plan (as per SOPs/WPDs of Sponsor's representative) describes all logical checks, qualitative checks, medical checks, manual checks and query processes used to clean the database and to assure accurate and consistent data in the database.

The Sponsor and the Sponsor's representative must approve all data handling and cleaning processes.

The Sponsor's representative will handle the data cleaning process, including logical checks, medical checks, and query processes.

After the complete CRF pages are collected and entered into the database and all the data cleaning activities are performed, the database will be locked when it is declared accurate and complete.

The database will be locked after the Blinded Data Review Meeting (BDRM) when it is declared complete and accurate. Sponsor approval prior to database lock is mandatory. Database lock is the prerequisite for the unblinding procedure.

.

For all laboratory variables described in Section 9.1.9, the pre-defined standard unit must be used.

Biomarker Data: *Data management of biomarker data will be described in the relevant laboratory manual. The final dataset will be transferred from RBM to PPD and from PPD to Takeda. The reconciliation and querying of the transferred laboratory data is covered in the Data Validation Manual.*

Metabolomics Data: *Data management of metabolomics data will be described in the relevant laboratory manual. The final dataset will be transferred from Metabolomics Health GmbH to PPD and from PPD to Takeda according to the Data Validation Manual.*

Coding Instructions: *Medical History and AEs will be coded according to Medical Dictionary for Regulatory Activities (MedDRA), concomitant medication according to the WHO Drug Dictionary (WHODD), and Anatomical Therapeutic Chemical (ATC) coding. The current version of the dictionaries will be used.*

The coding will be performed in accordance with the Coding described in the Data Validation Manual.

Revised text

The full details of procedures for data handling will be documented in the Data Management Plan. AEs, medical history, and concurrent conditions will be coded using the Medical Dictionary for Regulatory Activities (MedDRA [SOC, HLGT, HLT, LL, PT, and their corresponding descriptive terms]). Drugs will be coded using the World Health Organization (WHO) Drug Dictionary.

Rationale for Amendment

Remove detail from protocol and add wording to reflect that detailed information will be documented in the Data Management Plan.

Page 74, Section 13.1, Statistical and Analytical Plans

Existing text

A statistical analysis plan (SAP) will be prepared and finalized prior to unblinding of subject's treatment assignment. This document will provide further details regarding the definition of analysis variables and analysis methodology to address all study objectives. Data handling issues and exploratory analyses may be changed due to unforeseen reasons after the pre-BDRM(s) or BDRM before hard lock of the database followed by final production of analysis. All later deviations and/or alterations will be documented and summarised in the Clinical Trial Report.

Statistical analysis will be performed by the Sponsor's representative. The final statistical analysis must be approved by the Sponsor.

Revised text

A statistical analysis plan (SAP) will be prepared and finalized prior to unblinding of subject's treatment assignment. This document will provide further details regarding the definition of analysis variables and analysis methodology to address all study objectives. A data review, blinded for the main period, will be conducted prior to database lock Data handling issues and exploratory analyses may be changed due to unforeseen reasons after the blinded data review. All later deviations and/or alterations will be documented and summarised in the Clinical Trial Report.

Statistical analysis will be performed by the Sponsor's representative. The final statistical analysis must be approved by the Sponsor.

Rationale for Amendment

Clarify in line with new SOPs that blinded data review meetings no longer occur, however the data will be reviwed, in a blinded manner, prior to database lock.

Page 75, Section 13.1.1.4 Valid Cases Set

Existing text

Subjects with major protocol violations considering the inclusion criteria, randomization criteria, and criteria affecting the read-out parameters of the trial will be identified during the BDRM. Violation of the criteria within ethical considerations in terms of general health are considered minor deviations in terms of impact on interpretability of the data, but it will be assessed in the BDRM if there is an impact for single cases. Deviations occurring during the following procedures will not be considered major protocol violations for the primary objective of the trial: Telephone contacts after early withdrawal and follow-up visit after regular trial end.

Revised text

Subjects with major protocol violations considering the inclusion criteria, randomization criteria, and criteria affecting the read-out parameters of the trial will be identified during reviewof the blinded data. Violation of the criteria within ethical considerations in terms of general health are considered minor deviations in terms of impact on interpretability of the data, but it will be assessed during review of the blinded data if there is an impact for single cases. Deviations occurring during the following procedures will not be considered major protocol violations for the primary objective of the trial: Telephone contacts after early withdrawal and follow-up visit after regular trial end.

Rationale for Amendment

Clarify in line with new SOPs that blinded data review meetings no longer occur, however the data will be reviwed, in a blinded manner, prior to database lock.

<u>Page 91, Appendix A Schedule of Procedures</u>

Existing Text

Appendix M Schedule of Study Procedures

Activities and assessments	***Run-in period***			***Double-blind treatment period***				***FU safety visit*** [a)]
			R				***VE***	
Visit	***V0***	***V1***	***V2***	***V3***	***V4***	***V5***	***V6***	***V7***
Weeks	***-6***	***-2***	***0***	*≤2*	***6***	***14***	***16***	***≤18***
Visit Window (days) [b)]		*-2 / +3*	*-3 / +5*		*-3 / +5*	*-3 / +5*	*-7 / +7*	
Subject Information and Informed Consent	*X*							

Activities and assessments	***Run-in period***			***Double-blind treatment period***				***FU safety visit*** [a)]
Demographic data	*X*							
Medical History and concomitant diseases	*X*							
COPD history	*X*							
Inclusion/Exclusion criteria	*X*							
Withdrawal of disallowed medications	*X*							
Previous and concomitant medications	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Substance use (smoking status)	*X*	*X*	*X*		*X*	*X*	*X*	
Physical examination	*X*		*X*				*X*	*X*
Vital signs and body measurements (including body weight and body height) [c)]	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Subject fasting	*X*	*X*[d)]					*X*	
Standard laboratory (including blood pregnancy test) [e)]	*X*	*X*[d)]					*X*	
Urine analysis	*X*							
Urine pregnancy test [e)]		*X*	*X*		*X*	*X*		
Clotting time and platelet count	*X*	*X*				*X*		
Blood sampling for assessment of inflammatory biomarkers and metabolites in blood serum		*X*			*X*	*X*		
Sputum induction and sputum sample processing		*X*			*X*	*X*		
Bronchoscopy/bronchial biopsy			*X*				*X*	
Pulse oximetry measurement			*X*				*X*	
Chest X-ray or CT scan [f)] ***GERMANY ONLY:*** *Check of historical chest X-ray or CT scan* [f)]	*X*							
ECG (12-lead) at rest [g)]	*X*							
Pulmonary function test	*X*		*X*				*X*	
Randomization criteria check			*X*					
Randomization			*X*					
ID card dispensed	*X*							
Adverse event assessment (see Section 10.0)	*X*	*X*	*X*	*X*	*X*	*X*	*X*	*X*
Dispense trial treatment	*X*		*X*		*X*			
Trial treatment returned			*X*		*X*		*X*	
Tablet compliance check			*X*[h)]		*X*		*X*	

Activities and assessments	***Run-in period***			***Double-blind treatment period***				***FU safety visit*** [a)]
Termination of double-blind treatment phase of the trial							*X*	

Notes:

- V3 and V7, which are safety visits only, are to be conducted within two weeks after bronchoscopy. The exact timing remains at the discretion of the Investigator.
- Assessments described under V6 (VE) are mandatory for randomized Subjects upon trial completion either prematurely or as scheduled.
- In selected Subjects three protected brush specimen in a lower lobe bronchus will be collected at visits V2 and V6. This is described in a separate sub-trial protocol. With regard to Subject consent and information this sub-trial is independent from the RO-2455-402-RD trial protocol (see Section 9.1.15.3).

Abbreviations:
R - Randomization (V2); V0 - visit 0, VE - visit end (V6), FU safety visit - follow-up safety visit (V7)
a)Applies only to Subjects, who underwent the second bronchoscopy/bronchial biopsy at V6.
[b)] Time windows for the run-in period always refer to V0, whereas those for the treatment period always refer to the randomization visit V2.
[c)] Body height measurement only at visit V0.
[d)] If confirmation or check of V0 laboratory values is necessary. Pregnancy test is optional.
[e)] For female Subjects of childbearing potential or less than 1 year postmenopausal.
[f)] If not performed within past three months. Examination results must be available.
GERMANY ONLY: Every patient should have a historical X-ray or CT scan available to clarify the exclusion criteria #18.
[g)] This should be done prior to pulmonary function test.
[h)] Tablet compliance calculation by the investigational site (see Section 9.2) to check for randomization criterion no. 2 (see Section 7.3).

Revised Text

Appendix N Schedule of Study Procedures

Activities and assessments	Run-in period			Double-blind treatment period				FU safety visit [a)]
			R				VE	
Visit	V0	V1	V2	V3	V4	V5	V6	V7
Weeks	-6	-2	0	≤2	6	14	16	≤18
Visit Window (days) [b)]		-2 / +3	-3 / +5		-3 / +5	-3 / +5	-7 / +7	
Subject Information and Informed Consent	X							
Demographic data	X							
Medical History and concomitant diseases	X							
COPD history	X							
Inclusion/Exclusion criteria	X							
Withdrawal of disallowed medications	X							

Activities and assessments	Run-in period			Double-blind treatment period				FU safety visit [a)]
Previous and concomitant medications	X	X	X	X	X	X	X	X
Substance use (smoking status)	X	X	X		X	X	X	
Physical examination	X		X				X	X
Vital signs and body measurements (including body weight and body height) [c)]	X	X	X	X	X	X	X	X
Subject fasting, avoidance of strenuous exercise and smoking	**X**	**X[d)]**	**X**	**X***	**X***	**X***	**X**	**X***
Standard laboratory (including blood pregnancy test) [e)]	X	X[d)]					X	
Urine analysis	X							
Urine pregnancy test [e)]		X	X		X	X		
Clotting time and platelet count	X	X				X		
Blood sampling for assessment of inflammatory biomarkers and metabolites in blood serum		X			X	X		
Sputum induction and sputum sample processing		X			X	X		
Bronchoscopy/bronchial biopsy			X				X	
Pulse oximetry measurement			X				X	
Chest X-ray or CT scan [f)] *GERMANY ONLY:* Check of historical chest X-ray or CT scan [f)]	X							
ECG (12-lead) at rest [g)]	X							
Pulmonary function test	X		X				X	
Randomization criteria check			X					
Randomization			X					
ID card dispensed	X							
Adverse event assessment (see Section 10.0)	X	X	X	X	X	X	X	X
Dispense trial treatment	X		X		X			
Trial treatment returned			X		X		X	
Tablet compliance check			X[h)]		X		X	
Termination of double-blind treatment phase of the trial							X	

Notes:

- V3 and V7, which are safety visits only, are to be conducted within two weeks after bronchoscopy. The exact timing remains at the discretion of the Investigator.
- Assessments described under V6 (VE) are mandatory for randomized Subjects upon trial completion either prematurely or as scheduled.

- In selected Subjects three protected brush specimen in a middle lobe bronchus will be collected at visits V2 and V6. This is described in a separate sub-trial protocol. With regard to Subject consent and information this sub-trial is independent from the RO-2455-402-RD trial protocol (see Section 9.1.15.3).
- ***For visits where subjects will not undergo blood draws or biopsies, the fasting requirement will only be mandated if clinically indicated as per investigator judgement.**

Abbreviations:
R - Randomization (V2); V0 - visit 0, VE - visit end (V6), FU safety visit - follow-up safety visit (V7)
[a)] Applies only to Subjects, who underwent the second bronchoscopy/bronchial biopsy at V6.
[b)] Time windows for the run-in period always refer to V0, whereas those for the treatment period always refer to the randomization visit V2.
[c)] Body height measurement only at visit V0.
[d)] If confirmation or check of V0 laboratory values is necessary. Pregnancy test is optional.
[e)] For female Subjects of childbearing potential or less than 1 year postmenopausal.
[f)] If not performed within past three months. Examination results must be available.
GERMANY ONLY: Every patient should have a historical X-ray or CT scan available to clarify the exclusion criteria #18.
[g)] This should be done prior to pulmonary function test.
[h)] Tablet compliance calculation by the investigational site (see Section 9.2) to check for randomization criterion no. 2 (see Section 7.3).

Rationale for Amendment

Clarify requirement for subject to fast and avoid smoking and strenuous exercise prior to all study visits.