Acute and chronic inflammatory responses induced by smoking in individuals being susceptible and non-susceptible for development of COPD: from specific disease phenotyping towards novel therapy: a cross-sectional study

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Acute and chronic inflammatory responses induced by smoking in individuals being susceptible and non-susceptible for development of COPD: from specific disease phenotyping towards novel therapy: a cross-sectional study

Short title: Acute and chronic smoking effects and susceptibility to COPD

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ABSTRACT (*Word count = 300)*

Introduction: Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease with pulmonary and extra-pulmonary manifestations. Although COPD is a complex disease, the diagnosis and staging are still based on simple spirometry measurements. Different COPD phenotypes exist based on clinical, physiological, immunological, and radiological observations. Cigarette smoking is the most important risk factor for COPD, but only 15-20% of smokers develop the disease, suggesting a genetic predisposition. Unfortunately, little is known about the pathogenesis of COPD, and even less on the very first steps that are associated with an aberrant response to smoke exposure.

This study aims to investigate the underlying local and systemic inflammation of different clinical COPD phenotypes, and acute effects of cigarette smoke exposure in individuals susceptible and non-susceptible for the development of COPD. Furthermore, we will investigate mechanisms associated with corticosteroid insensitivity. Our study will provide valuable information regarding the pathogenetic mechanisms underlying the natural course of COPD.

Methods and analysis: This cross-sectional study will include young and old individuals susceptible or non-susceptible to develop COPD. At young age (18-40 years) 60 “party smokers” will be included that are called susceptible or non-susceptible based on COPD prevalence in smoking family members. Additionally, 30 healthy smokers (age 40-75 years) and 110 COPD patients will be included. Measurements will include questionnaires, pulmonary function, low-dose CT scanning of the lung, body composition, 6-min-walking distance, and biomarkers in peripheral blood, sputum, urine, exhaled breath condensate, epithelial lining fluid, bronchial brushes and biopsies. Non-biased approaches such as proteomics will be performed in blood and epithelial lining fluid.

Ethics and dissemination: This multicenter study was approved by the medical ethical committees of UMC Groningen and Utrecht, the Netherlands. The study findings will be presented at conferences and will be reported in peer-reviewed journals.

Trial registration: ClinicalTrials.gov, NCT00807469 (study 1) and NCT00850863 (study 2).

ARTICLE SUMMARY

Article focus

This article describes the study protocol of a cross-sectional study investigating the underlying local and systemic inflammation of different clinical COPD phenotypes, and acute effects of cigarette smoke exposure in individuals susceptible and non-susceptible for the development of COPD.

Key messages

- Young (18-40) and older (40-75) individuals who are susceptible and non-susceptible to develop COPD are included.
- All groups are extensively phenotyped by clinical, physiological, immunological, and radiographical characterisation. Furthermore, effects of acute smoking are studied.

Strengths and limitations of the study

- Extensive characterisation of a well defined study population, providing valuable information regarding the pathogenic mechanisms underlying the natural cause of COPD.
- Recruitment of a population with high and low familiar risk to develop COPD.

BACKGROUND

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide[1]. The disease is characterized by persistent and progressive expiratory airflow obstruction (post-bronchodilator $FEV_1/FVC<0.70$) and its severity is based on FEV_1 %predicted[1]. Cigarette smoking is the most important risk factor for COPD in the western world, but only 15-20 % of young smokers will eventually develop the disease, suggesting a genetic predisposition. So far, the genetic background of these susceptible smokers has not been elucidated[2]. Unravelling the underlying pathogenetic mechanisms of COPD is difficult because it takes 20-30 years of smoking before susceptible smokers develop established COPD. Also, there is the problem that COPD has many clinical expressions, and we just have started to learn how to phenotype this heterogeneous disease. Finally, there are many other risk factors that may modulate the complex interaction between the genetic background and smoking, like *in utero* events, microbial infections, dietary factors, physical inactivity and pharmacological treatment.

It is well accepted that the spirometry measurements (FEV_1 and FVC) are largely insufficient to diagnose and classify COPD[1]. With the increased recognition of the various clinical expressions of COPD, consensus is growing that COPD represents a spectrum of overlapping diseases with important extra-pulmonary consequences. Phenotypes of COPD may be classified according to four domains: clinical, physiological, immunological and radiographical[3].

- Clinical distinctions are generally based on dyspnea scores, frequency of exacerbations, body mass, muscle wasting, corticosteroid responsiveness, depression / anxiety, co-morbidity, and healthy status[4].
- Physiological distinctions may be based on the degree of airflow limitation, decline in lung function, bronchodilator responsiveness, airway hyperresponsiveness, CO diffusion capacity, hyperinflation, body-plethysmography, bio-impedance, and exercise tolerance.

- Immunological features comprise the type and severity of local and systemic immunological processes in the lung and systemic compartment. In blood leukocytes cytokines, and mediators may affect the functionality of extra-pulmonary tissues and organs, leading to COPD-associated co-morbid conditions.
- Radiographic distinctions may be based on the presence of various forms and severity of emphysema, thickened large airways, and small airways abnormality on high-resolution computed tomography scans.

Although systemic inflammation and multi-organ pathology have been put forward as important features of COPD, surprisingly little is known about the underlying pathogenesis. Most COPD studies in this field included small numbers of individuals, focused on more severe stages of COPD, characterized subjects clinically on the basis of few arbitrary pulmonary measurements, did not take into account the genetic background and paid limited attention to different aspects of systemic inflammation. In addition, most studies assumed that assessment of cytokines by multiplex assays (e.g. Luminex) is sufficient to accurately describe the systemic inflammatory response. Unfortunately, many caveats are present that preclude a complete insight in this response, e.g.:

- not all cytokines implicated in COPD are known,
- little effort is taken to measure anti-inflammatory cytokines (the balance between pro- and anti-inflammatory signals will probably determine the extent and type of inflammation),
- different pro-inflammatory cytokines can act as heterologous antagonists (inhibit the effects of other cytokines).
- the kinetics of cytokines is very dynamic and no consensus is present regarding an optimal single time point for blood collection.

In the present study we set out to characterize systemic inflammation by an alternative approach. Innate immune cells will be used as integrators of pro- and anti-inflammatory

signals. We hypothesize that subtle changes in the phenotype of granulocytes and monocytes are caused by an “inflammatory imprinting” of these cells.

Cigarette smoking is the main risk factor for developing COPD. Repetitive acute effects of cigarette smoke exposure may accumulate and after many years lead to irreversible lung damage. To understand the changes in the lung due to chronic smoking we believe that it is important to first investigate the exact immunological responses to an acute smoke exposure event, particularly in “naive” lungs that are not yet affected by chronic smoke exposure. The acute (<24 hours) effects of smoking in humans, animals and cell cultures have been extensively reviewed some years ago by van der Vaart and colleagues[5]. If we integrate all available data on acute smoking we are able to construct a hypothetical time frame for the acute effects of smoking (figure 1). One of the very first insults on the bronchial system is by oxidants present in cigarette smoke. After local depletion of anti-oxidants, the first oxidative stress products can be measured within 1 hour. These products will disappear within 6 hours. There is a surprisingly fast influx of inflammatory cells; even faster than the synthesis of some pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-8). The exact time period at which the proteinase / antiproteinase balance is affected is unknown; however, protein degradation is measurable within 6 hours after smoking. Unfortunately, until now, only a few studies have investigated the acute effects of cigarette smoking in humans[5]. These studies included only small numbers of individuals, characterized subjects mainly on basis of pulmonary measurements, paid no attention to the genetic background and paid limited attention to different aspects of pulmonary inflammation.

Corticosteroids provide little therapeutic benefit in a relatively large group of COPD patients, despite their broad anti-inflammatory effects. Our goal is to identify common markers in peripheral blood monocytes, skin and lung epithelial cells that might contribute to corticosteroid insensitivity. Recently, the GLUCOLD study demonstrated beneficial effects on airway wall inflammation and decline in lung function yet with large inter-individual

differences[6]. *In vitro* studies have shown that the ability of dexamethasone to suppress cytokine release (e.g. IL-8) from alveolar macrophages is impaired in COPD patients as compared to healthy smokers[7]. Furthermore, alveolar macrophages from healthy smokers are more resistant to corticosteroids than macrophages from non-smokers[8]. This relative steroid insensitivity may, in part, be explained by a suppressive effect of cigarette smoke-induced oxidative stress. This suppression may particularly play a role in the airway epithelium, where cells are in first contact with cigarette smoke and form an important source of mediators involved in the induction of neutrophilic airway inflammation (e.g. the chemoattractant IL-8). It may well be that corticosteroid insensitivity is gradually acquired by smoking in COPD, and one might hypothesize that smokers who develop COPD are more prone to have signs of corticosteroid insensitivity.

General hypotheses

There is a clear need to better understand all factors that contribute to the development of COPD and its different phenotypes. This study focuses on the pathogenesis and clinical expression of smoking-induced COPD, studied both in the pulmonary and the systemic compartments. The following general hypothesis is put forward by our consortium (figure 2):

"COPD is a multi-organ disease situated in both the lung and extra-pulmonary organs and tissues. Dysfunction of the latter tissues is exemplified by muscle atrophy, impaired muscle oxidative capacity, osteoporosis, atherosclerosis and heart failure. A low-grade systemic inflammation plays a pivotal role in the induction and perpetuation of this multi-organ disease. Smoking and persistent production of inflammatory mediators from the lung are inducers of systemic inflammation. Other risk factors such as diet deficiencies, sedentary life style, and frequent infections contribute independently to further amplification of systemic inflammation. In more advanced COPD the extra-pulmonary pathology starts to contribute to disease severity and a vicious circle of persistent difficulty to treat inflammation. Consequently, local and systemic inflammation should be reduced in all stages of disease by

reversing negative life style factors and applying successful anti-inflammatory treatment modalities. In more advanced stages multimodal interventions additionally should improve impaired tissue functions. An important contributing problem is the relative corticosteroid insensitivity of both lung and peripheral tissue responses in COPD."

Aims of the study

- To assess systemic and local inflammation at baseline in:
    a) young healthy individuals with low number of pack years smoking who have a high and low familial risk to develop COPD;
    b) older individuals with higher number of pack years who either have normal lung function or COPD.

  We hypothesize that young susceptible individuals and COPD patients demonstrate a higher degree and different type of local and systemic inflammation at baseline.
- To study systemic and local inflammation after acute smoke exposure in the above groups. We hypothesize that young susceptible individuals and COPD patients demonstrate a higher and aberrant local and systemic inflammatory response to cigarette smoke.
- To compare in bronchial epithelial cells and PBMCs corticosteroid responsiveness *in vitro* between susceptible and non-susceptible individuals. To study in these cells the effects of cigarette smoking and to elucidate underlying mechanisms of corticosteroid unresponsiveness.
- To determine whether the type and severity of the systemic inflammatory response contributes to the clinical outcome of COPD. We hypothesize that the type and severity of systemic inflammation have profound effects on the clinical picture of COPD.
- To investigate the relationship between downstream genetic effects (transcriptome, proteome) and specific COPD phenotypes in peripheral blood and lung tissue (induced sputum, bronchial biopsies, epithelial lining fluid).

METHODS

Study population

In total 200 old and young individuals who are susceptible or not susceptible to develop COPD will be recruited (table 1). At old age (>40 years), 30 healthy smokers (>20 pack years) and 110 COPD patients (>10 pack years) will be enrolled in the study. At young age, 60 “party smokers” with a normal lung function will be included with a high or low prevalence of COPD in smoking family members (see table 1). Party smoking was defined by irregularly smoking and/or able to quit smoking for at least two days. Exclusion criteria are: α-1-anti-trypsin-deficiency, acute pulmonary infections (like tuberculosis, pneumonia, flue, tracheo-bronchitis), prior history of significant inflammatory lung disease other than COPD (sarcoidosis, pulmonary fibrosis, silicosis, ect.), active infections (such as hepatitis A-C, cystitis, gastro-enteritis etc.), treatment with antibiotics or corticosteroids within 8 weeks, taking part in another study, recent diagnosis of cancer. Medication such as NSAIDs and immunosuppressive agents which could affect the results of the study will be excluded, as well as substance abuse. Co-morbidities that might lead to study-related (serious) adverse events will be excluded on basis of an arbitrary selection of conditions listed in the ACE-27 co-morbidity scale[9].

Table 1 Study population

Disease	No	Age (Yrs)	Smoking status	Pack years	FEV_1 /VC,%	FEV_1, % pred
Non-susceptible						
Healthy [A]	30	18-40	Party smoking	0-10	> 70	> 85
Healthy [B]	30	40-75	Ex or current	>20	> 70	> 85
Susceptible						
Healthy [C]	30	18-40	Party smoking	0-10	> 70	> 85
COPD						
Stage I [D1]	30	40-75	Ex or current	>10	≤ 70	> 80
Stage II [D2]	30	40-75		>10	≤ 70	50-80
Stage III [D3]	30	40-75		>10	≤ 70	30-50
Stage IV [D4]*	20	40-75		>10	≤ 70	< 30
		< 53				< 30%

*Susceptibility in young individuals is based on family history. Not susceptible means that none of the smoking family members who are at least 40 years of age have COPD. Susceptible means that the prevalence of COPD in smoking family members older than 40 years is high: 2 out of 2, 2 out of 3 or 3 out of 3, 3 out of 4 or 4 out of 4. Party smoking was defined by irregularly smoking and/or able to quit smoking for at least two days. Alpha-1-antitrypsin deficiency is excluded. *patients with a FEV_1 30-50% predicted in combination with chronic respiratory failure also have stage IV.*

Study design

This study is a bi-center cross-sectional study that takes place at the University Medical Centers Utrecht (UMC Utrecht) and Groningen (UMC Groningen). Participating subjects will undergo extensive clinical characterisation (table 2). Local and systemic inflammation will be investigated in several ways. Special attention will be paid to acute smoking and corticosteroid insensitivity in selected subgroups.

Table 2 Measurements

	Measurements	Group
Clinical	Demographics	All
	Physical examination	All
	Peripheral blood (routine measurements)	All
	Presence of metabolic syndrome	All
	ECG	B, D1-4
	Bode index	B, D1-4
	Fagerstrom Smoking Questionnaire	All
	St Georges Respiratory Questionnaire (SGRQ)	D1-4
	Clinical COPD Questionnaire (CCQ)	D1-4
	SQUASH	All
	Urine (microproteins)	All
	AGE (Advanced Glycation Endproducts)-reader	All
	Skin blanching test	All
Physiological	Flow volume + reversibility	All
	Body plethysmography	All
	CO diffusion	All
	Methacholine challenge test	A,B,C,D1-3
	Bioelectrical impedance	All
	Six minute walking distance	B,D1-4
Immunological	Sputum induction (only baseline)	A,B,C,D2
	Peripheral blood (systemic inflammation)	All
	Peripheral blood 4x (acute smoking)	A,B,C,D2
	Exhaled breath condensate 3x (acute smoking)	A,B,C,D2
	Exhaled CO 5x (acute smoking)	A,B,C,D2
	Bronchial biopsy 2x (acute smoking)	A,B,C,D2
	Epithelial lining fluid 2x (acute smoking)	A,B,C,D2
	Epithelial brushes 2x (acute smoking)	A,B,C,D2
Radiographical	Low dose HRCT-scan lung	All

Clinical outcomes

Demographic variables include: age, sex, smoking habits, education, profession, other exposures, height and weight. Risk factors of the metabolic syndrome will be determined including blood pressure, waist hip circumference, lipid profile and fasting glucose (table 2). Questionnaires will be the Clinical COPD Questionnaire (CCQ), the St Georges Respiratory Questionnaire (SGRQ), the Dutch Fagerstrom test for nicotine dependence, and the Short QUestionnaire to ASsess Health-enhancing physical activity (SQUASH)[10-12]. Exacerbation frequency will be recorded in COPD patients. The BODE-index will be calculated on basis of FEV_1, six-minute-walking distance, Body Mass Index (BMI) and MRC-dyspnea score[4]. In urine (micro) protein concentration will be assessed. Corticosteroid sensitivity will be measured by the cutaneous vasoconstrictor response to topical budesonide using the skin blanching test[13]. Budesonide dissolved in 95% ethanol will be applied to the skin using eight different concentrations (0-1000 µg/ml). Blanching will be scored with a 7-point scale: 0-3 (increasing with steps of 0.5; 0 = no blanching and 3 = intense blanching). Cumulative oxidative stress will be measured in the skin using the non-invasive AGE (Advanced Glycation Endproducts) reader (DiagnOptics, Groningen, The Netherlands)[14].

Physiological outcomes

Spirometry will be performed according to international guidelines (ERS 2005)[15]. We will assess FEV_1, FEV_1/FVC, IVC, FEF50, FEF75, reversibility to salbutamol, TLC, FRC (body box), and CO diffusion. Methacholine challenge tests are performed according to international guidelines (ERS 2005), using serial doubling concentrations of methacholine-bromide (0.03 to 38.4 mg/ml) with the 2-min tidal breathing method at 5-minute intervals. The six-minute-walking-distance (6MWD) will be determined according the American Thoracic Society published guidelines of 2002[16]. Individuals should walk at their own pace, can stop if necessary, and are allowed to use oxygen. Body composition will be estimated using single frequency (50 kHz) bioelectrical impedance (Biostat 500), and fat-free-mass will be calculated with the disease-specific equation of Schols et al[17].

Immunological outcomes

Lung inflammation

- Sputum will be induced and processed according to a validated and standardized technique[18], with some modifications. Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells will be performed on May Grünwald Giemsa (MGG) stained cytospins by a qualified cytopathologist.
- Exhaled breath condensate (EBC) will be collected using The EcoScreen® (Jaeger, Hoechberg, Germany). Hydrogen peroxide, pH, 8-isoprostane, nitrite, nitrate, 4-hydroxy-2-nonenal and malondialdehyde will be measured.
- Bronchoscopy will be performed using established guidelines[19-21], and 6 bronchial biopsies will be taken from subsegmental carinae in the right or left lower lobe. Epithelial morphology, epithelial proliferation, and basement membrane thickness will be measured[22]. Submucosal density of inflammatory cells (AA1, EG2, CD68, CD3, CD4, CD4CD25, CD8, mast cells, neutrophils) will be quantitated in a semi-automated way[22]. Expression of E-Cadherin, VEGF, ICAM, VCAM, E-selection, P-selectin, AGEs and RAGEs will be measured.
- Epithelial lining fluid will be sampled by advancing 3 microsample probes (BC -401C, Olympus, Tokyo, Japan) in the lumen of the left main bronchus[23,24]. Cytokines will be measured by Luminex (Linco, Nuclilab BV, Ede, The Netherlands). 90% of the ELF will be used for proteomic analysis. Briefly, each trypsin digested sample will be labeled (iTRAQ® Reagent 8-plex, ABSciex, Foster City, CA, USA) according to the manufacturer's protocol. The individually labeled digests will be combined into a single sample mixture and subjected to strong-cation exchange chromatography (AKTA Purifier, GE Healthcare Biosciences AB, Uppsala, Sweden). The resulting peptide-containing fractions will be separated by reversed-phase chromatography (Ultimate 3000 nanoflow liquid chromatography system, Dionex, Amsterdam, The Netherlands). Fractions of 12 sec will be spotted on MALDI targets (Probot, Dionex, Amsterdam, The Netherlands) and mass spectrometric analysis will be carried out on a 4800 Proteomics Analyzer MALDI

TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA) controlled by the 4000 Series Explorer v3.5 software. Proteins will be identified using Protein Pilot® software v2.0 (Applied Biosystems).

- Bronchial epithelial cells will be harvested from the right or left main bronchus by brushing as described elsewhere[25]. Brushed epithelial cell will be cultured to enable corticosteroid sensitivity experiments.

Systemic inflammation

Systemic inflammation will be measured in peripheral blood using several methods to study systemic activation of innate immune cells at three different levels:

- Expression of established and newly markers on innate immune cells associated with pre-activation[26,27]
- Determination of the sensitivity of innate immune cells for stimuli
- Genomic and proteomic analysis of innate immune cells in vivo[28]
- Multiplex analysis of the presence of pro-and anti-inflammatory cytokines in plasma/serum.

Systemic inflammation will also be measured in peripheral blood using peripheral blood mononuclear cells (PBMC's):

- Expression of intracellular and cell-surface markers of adaptive immune cells (Th1-cells, Th2-cells, Th17-cells, T_{reg}-cells, B-cells, NK-cells)

Lung and systemic inflammation after acute smoking

Young and old subjects who are susceptible or not susceptible to develop COPD will smoke 3 cigarettes in 1 hour. Blood samples, urine, exhaled breath condensate, bronchial biopsies, epithelial lining fluid and epithelial brushes will be collected at baseline and after smoking according the scheme in table 3. Exhaled CO will be measured at baseline to check if individuals did not smoke recently, and after smoking to check if individuals inhaled cigarette smoke sufficiently.

Sputum supernatant, epithelial cells, serum, plasma, DNA, and RNA of blood will be stored for further analyses.

Table 3 Acute smoke model

	Baseline	Smoking 3 cigarettes	5 minutes	2 hours	24 hours	6 weeks
Exhaled CO	x		x	x	x	x
Blood	x			x		
Exhaled breath condensate	x		x	x		
Urine	x			x	x	
Bronchial Biopsies					x	x
Epithelial brush					x	x
Microsampling probe (ELF)					x	x

Radiological outcomes

All subjects will undergo a low-dose CT-scan at full inspiration and expiration. Exposure settings will be 30 mAs at 90 kVp for patients weighing less then 50 kg, 30 mAs at 120 kVp for patients weighing between 50 and 80 kg and 30 mAs at 140 kVp for those weighing more than 80 kg without dose modulation. During expiration the exposure settings will be 20 mAs at 90 kVp (body mass < 80kg) or 20 mAs at 120 kVp (body mass > 80kg). Emphysematous lung changes will be quantitated using automated software on low-dose CT scanning images developed in the UMC Utrecht.

Ethics and dissemination

The two studies are registered at clinicaltrial.gov (identifier study 1: NCT00807469 and identifier study 2: NCT 00850863). These two studies have been judged by the medical ethical committee of UMC Groningen and additionally study 2 has been minimally judged by

the medical ethical committee of UMC Utrecht. The study findings will be presented at conferences and will be reported in peer-reviewed journals.

DISCUSSION

There is a large backlog in the recognition of different phenotypes of COPD and their underlying immunopathological processes. This importantly hinders the appropriate diagnosis, treatment and prognosis of this disabilitating disease. Currently lung function (FEV_1 and FEV_1/FVC) is still the standard for the diagnosis and classification of COPD[1]. However, there is general consensus that FEV_1 poorly correlates with important patient-centred outcomes such as quality of life, symptoms and exercise capacity[29]. Celli et al showed an association between FEV_1 and mortality when FEV_1 was combined with MRC-dyspnoea score, 6-minute walking distance and BMI. The so-called BODE index was put forward as a composite measure to characterise COPD in a more realistic way[4]. In the last decades different approaches have been put forward to characterize COPD leading to at least 16 different phenotypes[30]. Although clinically relevant in terms of presentation, triggers and treatment response these phenotypes do not necessarily give insight into the underlying disease processes of COPD. In this perspective the term intermediate phenotype or endotype has been put forward to describe a subtype of a disease which is defined by a distinct functional or pathophysiological mechanism[31]. Together with genetic and environmental factors intermediate phenotypes may explain the clinical presentation of a heterogeneous disease like COPD. Accordingly, the present study will phenotype the induction and progression of COPD and associate this with underlying pathophysiological mechanisms in a biased as well as non-biased way. As smoking is the most important environmental risk factor for COPD we will use an acute smoking model to evaluate differences in smoking-induced acute mechanisms differentially expressed between individuals with a high and low risk for development of COPD.

Recently, a large prospective cohort study (ECLIPSE) was initiated to study the natural course of COPD in order to gain more insight in the underlying pathogenetic mechanisms[32]. The ECLIPSE study is a three year observational study including current and ex-smoking COPD patients and healthy controls with and without a smoking history.

Indeed the ECLIPSE study confirmed that the clinical manifestations of COPD are highly variable and that the degree of airflow limitation does not capture the heterogeneity of the disease[33]. Particularly, the rate of change in FEV_1 among patients with COPD was highly variable, with increased rates of decline among current smokers, patients with bronchodilator reversibility and with emphysema[34]. Several new susceptibility genes have been identified in the ECLIPSE study[35,36], as well as potentially useful biomarkers[37-39]. However, in contrast to our study ECLIPSE does not include young subjects and is, therefore, not able to investigate the susceptibility for COPD at young age. ECLIPSE investigates aspects of systemic inflammation (CRP, TNF-α, IL-6, IL-8, SDP), but does not investigate the activation state of circulating neutrophils and lymphocytes, nor does it perform unbiased proteomic analyses of epithelial lining fluid and peripheral blood neutrophils. Therefore, our study will complement ECLIPSE data by focusing on the pathogenesis of local and systemic inflammation by using unique approaches to link genomic and inflammatory phenotypes in all stages of COPD from preclinical to advanced disease.

The present study has already been started and recruitment is still ongoing. The study population has been described in ClinicalTrial.gov (NCT00807469, NCT 00850863) and was divided into 9 groups. Initially, we planned to distinguish susceptible individuals into a “susceptible” and “very susceptible” group. The group of “old” very susceptible individuals should include early-onset COPD (FEV_1/FVC<70%, FEV_1< 40% predicted, age<53 years) and COPD with low number of pack years (FEV_1/FVC < 70%, FEV_1 < 80%predicted, pack years<5). The group of “young” very susceptible individuals should have included young individuals with family members with early-onset COPD or COPD with low smoke exposure. Despite an intensive search among lung transplantation (LTx) candidates/recipients and their family members we were not able to recruit this group in sufficiently high numbers. Therefore, we decided to combine the susceptible and very susceptible groups.

COPD is often accompanied by different co-morbidities, especially cardiovascular conditions, which also affect the prognosis of the disease as well as quality of life and cost of COPD[40,41]. Consequently, we do not exclude subjects with cardiovascular co-morbidity conditions unless the condition was acute or too severe. We use the selected grade 1-3 co-morbidity list in the ACE-27[9] to exclude patients with co-morbidities within grade 2 or 3 in all organ systems except the respiratory system. We also exclude subjects with systemic inflammatory diseases such as rheumatoid arthritis, because we might investigate systemic inflammation related to other systemic inflammatory diseases.

In conclusion this study will provide valuable information regarding the pathogenetic mechanisms underlying the development of COPD, which in the future will help us to develop new targets for the management of different phenotypes of COPD.

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Competing Interests

AT, SH, LF, RB, RH, IH, AO, MB, JWL, and LK have no competing interests to declare. WT has received payment for lectures from Chiesi, GSK, and Roche diagnostics. DP has received an unrestricted educational grant for research from AstraZeneca and Chiesi; fees for consultancies by DP were given to the University of Groningen by AZ, Boehringer Ingelheim, Chiesi, GSK, Nycomed and TEVA; NH received grants from GlaxoSmithKline, Boehringer Ingelheim, Nycomed and Chiesi.

Authors' contributions

NH, JWL, DSP, LK, RB, AO, WT, and MB participated in the design and supervision of the study. ALT and SH were involved in the patient-related investigations and contributed equally to the manuscript. LF performed the proteomic analyses of epithelial lining fluid. RH investigated in vitro corticosteroid sensitivity of epithelial cells. All authors read and approved the final manuscript.

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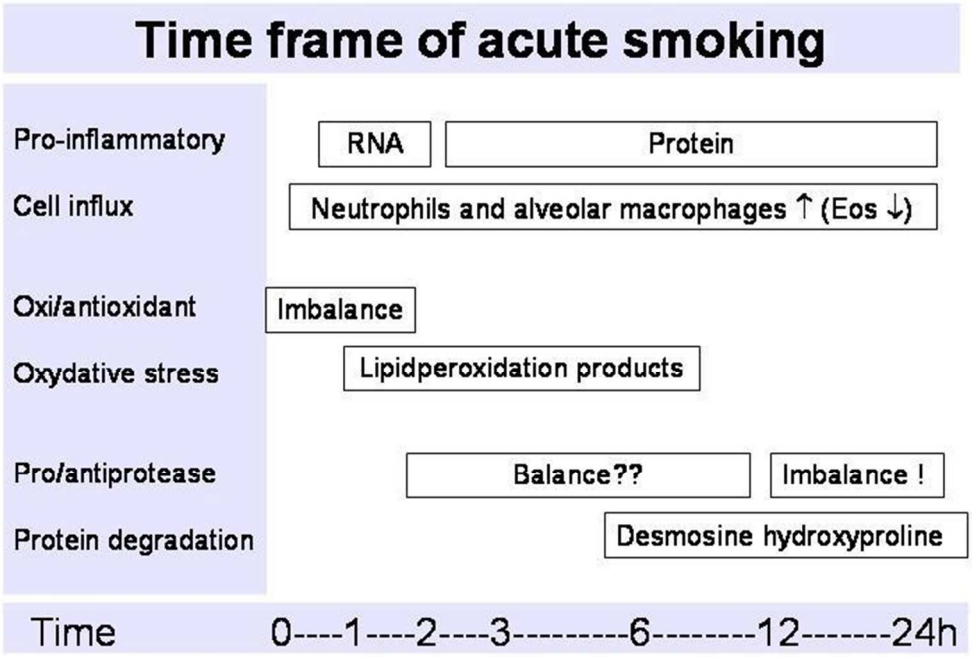
Figure 1 Acute smoking effects in the lung

Figure 2 General hypothesis about the role of systemic inflammation



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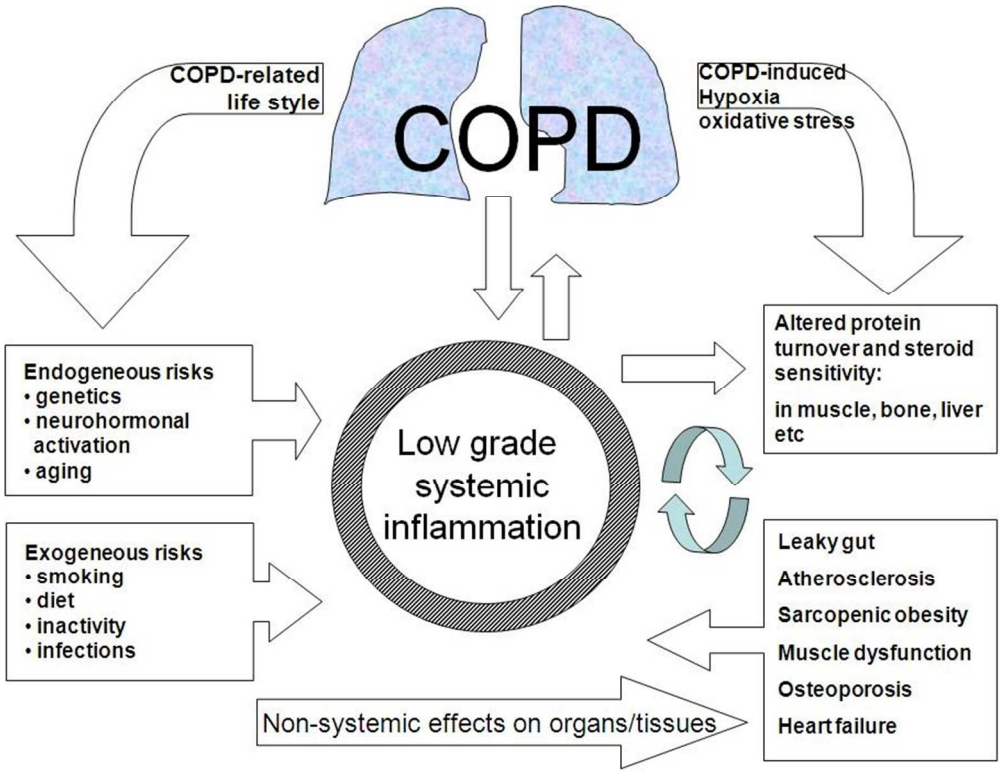

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Acute and chronic inflammatory responses induced by smoking in individuals being susceptible and non-susceptible for development of COPD: from specific disease phenotyping towards novel therapy: protocol of a cross-sectional study

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Primary Subject	Respiratory medicine

Heading:	
Secondary Subject Heading:	Smoking and tobacco
Keywords:	COPD, Inflammation, Susceptibility, Corticosteroid insensitivity, Smoking

Acute and chronic inflammatory responses induced by smoking in individuals being susceptible and non-susceptible for development of COPD: from specific disease phenotyping towards novel therapy: protocol of a cross-sectional study

Short title: Acute and chronic smoking effects and susceptibility to COPD

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ABSTRACT (*Word count = 300)*

Introduction: Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease with pulmonary and extra-pulmonary manifestations. Although COPD is a complex disease, the diagnosis and staging are still based on simple spirometry measurements. Different COPD phenotypes exist based on clinical, physiological, immunological, and radiological observations. Cigarette smoking is the most important risk factor for COPD, but only 15-20% of smokers develop the disease, suggesting a genetic predisposition. Unfortunately, little is known about the pathogenesis of COPD, and even less on the very first steps that are associated with an aberrant response to smoke exposure.

This study aims to investigate the underlying local and systemic inflammation of different clinical COPD phenotypes, and acute effects of cigarette smoke exposure in individuals susceptible and non-susceptible for the development of COPD. Furthermore, we will investigate mechanisms associated with corticosteroid insensitivity. Our study will provide valuable information regarding the pathogenetic mechanisms underlying the natural course of COPD.

Methods and analysis: This cross-sectional study will include young and old individuals susceptible or non-susceptible to develop COPD. At young age (18-40 years) 60 “party smokers” will be included that are called susceptible or non-susceptible based on COPD prevalence in smoking family members. Additionally, 30 healthy smokers (age 40-75 years) and 110 COPD patients will be included. Measurements will include questionnaires, pulmonary function, low-dose CT scanning of the lung, body composition, 6-min-walking distance, and biomarkers in peripheral blood, sputum, urine, exhaled breath condensate, epithelial lining fluid, bronchial brushes and biopsies. Non-biased approaches such as proteomics will be performed in blood and epithelial lining fluid.

Ethics and dissemination: This multicenter study was approved by the medical ethical committees of UMC Groningen and Utrecht, the Netherlands. The study findings will be presented at conferences and will be reported in peer-reviewed journals.

Trial registration: ClinicalTrials.gov, NCT00807469 (study 1) and NCT00850863 (study 2).

ARTICLE SUMMARY

Article focus

This article describes the study protocol of a cross-sectional study investigating the underlying local and systemic inflammation of different clinical COPD phenotypes, and acute effects of cigarette smoke exposure in individuals susceptible and non-susceptible for the development of COPD.

Key messages

- Young (18-40) and older (40-75) individuals who are susceptible and non-susceptible to develop COPD are included.
- All groups are extensively phenotyped by clinical, physiological, immunological, and radiographical characterisation. Furthermore, effects of acute smoking are studied.

Strengths and limitations of the study

- Extensive characterisation of a well defined study population, providing valuable information regarding the pathogenic mechanisms underlying the natural cause of COPD.
- Recruitment of a population with high and low familiar risk to develop COPD.

BACKGROUND

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide(1). The disease is characterized by persistent and progressive expiratory airflow obstruction (post-bronchodilator $FEV_1/FVC<0.70$) and its severity is based on FEV_1 %predicted(1). Cigarette smoking is the most important risk factor for COPD in the western world, but only 15-20 % of young smokers will eventually develop the disease, suggesting a genetic predisposition. So far, the genetic background of these susceptible smokers has not been elucidated(2). Unravelling the underlying pathogenetic mechanisms of COPD is difficult because it takes 20-30 years of smoking before susceptible smokers develop established COPD. Also, there is the problem that COPD has many clinical expressions, and we just have started to learn how to phenotype this heterogeneous disease. Finally, there are many other risk factors that may modulate the complex interaction between the genetic background and smoking, like *in utero* events, microbial infections, dietary factors, physical inactivity and pharmacological treatment.

It is well accepted that the spirometry measurements (FEV_1 and FVC) are largely insufficient to diagnose and classify COPD(1). With the increased recognition of the various clinical expressions of COPD, consensus is growing that COPD represents a spectrum of overlapping diseases with important extra-pulmonary consequences. Phenotypes of COPD may be classified according to four domains: clinical, physiological, immunological and radiographical(3).

- Clinical distinctions are generally based on dyspnea scores, frequency of exacerbations, body mass, muscle wasting, corticosteroid responsiveness, depression / anxiety, co-morbidity, and healthy status(4).
- Physiological distinctions may be based on the degree of airflow limitation, decline in lung function, bronchodilator responsiveness, airway hyperresponsiveness, CO diffusion capacity, hyperinflation, body-plethysmography, bio-impedance, and exercise tolerance.

- Immunological features comprise the type and severity of local and systemic immunological processes in the lung and systemic compartment. In blood leukocytes cytokines, and mediators may affect the functionality of extra-pulmonary tissues and organs, leading to COPD-associated co-morbid conditions.
- Radiographic distinctions may be based on the presence of various forms and severity of emphysema, thickened large airways, and small airways abnormality on high-resolution computed tomography scans.

Although systemic inflammation and multi-organ pathology have been put forward as important features of COPD, surprisingly little is known about the underlying pathogenesis. Most COPD studies in this field included small numbers of individuals, focused on more severe stages of COPD, characterized subjects clinically on the basis of few arbitrary pulmonary measurements, did not take into account the genetic background and paid limited attention to different aspects of systemic inflammation. In addition, most studies assumed that assessment of cytokines by multiplex assays (e.g. Luminex) is sufficient to accurately describe the systemic inflammatory response. Unfortunately, many caveats are present that preclude a complete insight in this response, e.g.:

- not all cytokines implicated in COPD are known,
- little effort is taken to measure anti-inflammatory cytokines (the balance between pro- and anti-inflammatory signals will probably determine the extent and type of inflammation),
- different pro-inflammatory cytokines can act as heterologous antagonists (inhibit the effects of other cytokines).
- the kinetics of cytokines is very dynamic and no consensus is present regarding an optimal single time point for blood collection.

In the present study we set out to characterize systemic inflammation by an alternative approach. Innate immune cells will be used as integrators of pro- and anti-inflammatory

signals. We hypothesize that subtle changes in the phenotype of granulocytes and monocytes are caused by an “inflammatory imprinting” of these cells.

Cigarette smoking is the main risk factor for developing COPD. Repetitive acute effects of cigarette smoke exposure may accumulate and after many years lead to irreversible lung damage. To understand the changes in the lung due to chronic smoking we believe that it is important to first investigate the exact immunological responses to an acute smoke exposure event, particularly in “naive” lungs that are not yet affected by chronic smoke exposure. The acute (<24 hours) effects of smoking in humans, animals and cell cultures have been extensively reviewed some years ago by van der Vaart and colleagues(5). If we integrate all available data on acute smoking we are able to construct a hypothetical time frame for the acute effects of smoking (figure 1). One of the very first insults on the bronchial system is by oxidants present in cigarette smoke. After local depletion of anti-oxidants, the first oxidative stress products can be measured within 1 hour. These products will disappear within 6 hours. There is a surprisingly fast influx of inflammatory cells; even faster than the synthesis of some pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-8). The exact time period at which the proteinase / antiproteinase balance is affected is unknown; however, protein degradation is measurable within 6 hours after smoking. Unfortunately, until now, only a few studies have investigated the acute effects of cigarette smoking in humans(5). These studies included only small numbers of individuals, characterized subjects mainly on basis of pulmonary measurements, paid no attention to the genetic background and paid limited attention to different aspects of pulmonary inflammation.

Corticosteroids provide little therapeutic benefit in a relatively large group of COPD patients, despite their broad anti-inflammatory effects. Our goal is to identify common markers in peripheral blood monocytes, skin and lung epithelial cells that might contribute to corticosteroid insensitivity. Recently, the GLUCOLD study demonstrated beneficial effects on airway wall inflammation and decline in lung function yet with large inter-individual

differences(6). *In vitro* studies have shown that the ability of dexamethasone to suppress cytokine release (e.g. IL-8) from alveolar macrophages is impaired in COPD patients as compared to healthy smokers(7). Furthermore, alveolar macrophages from healthy smokers are more resistant to corticosteroids than macrophages from non-smokers(8). This relative steroid insensitivity may, in part, be explained by a suppressive effect of cigarette smoke-induced oxidative stress. This suppression may particularly play a role in the airway epithelium, where cells are in first contact with cigarette smoke and form an important source of mediators involved in the induction of neutrophilic airway inflammation (e.g. the chemoattractant IL-8). It may well be that corticosteroid insensitivity is gradually acquired by smoking in COPD, and one might hypothesize that smokers who develop COPD are more prone to have signs of corticosteroid insensitivity.

General hypotheses

There is a clear need to better understand all factors that contribute to the development of COPD and its different phenotypes. This study focuses on the pathogenesis and clinical expression of smoking-induced COPD, studied both in the pulmonary and the systemic compartments. The following general hypothesis is put forward by our consortium (figure 2):

"COPD is a multi-organ disease situated in both the lung and extra-pulmonary organs and tissues. Dysfunction of the latter tissues is exemplified by muscle atrophy, impaired muscle oxidative capacity, osteoporosis, atherosclerosis and heart failure. A low-grade systemic inflammation plays a pivotal role in the induction and perpetuation of this multi-organ disease. Smoking and persistent production of inflammatory mediators from the lung are inducers of systemic inflammation. Other risk factors such as diet deficiencies, sedentary life style, and frequent infections contribute independently to further amplification of systemic inflammation. In more advanced COPD the extra-pulmonary pathology starts to contribute to disease severity and a vicious circle of persistent difficulty to treat inflammation. Consequently, local and systemic inflammation should be reduced in all stages of disease by

reversing negative life style factors and applying successful anti-inflammatory treatment modalities. In more advanced stages multimodal interventions additionally should improve impaired tissue functions. An important contributing problem is the relative corticosteroid insensitivity of both lung and peripheral tissue responses in COPD."

Aims of the study

- To assess systemic and local inflammation at baseline in:
    a) young healthy individuals with low number of pack years smoking who have a high and low familial risk to develop COPD;
    b) older individuals with higher number of pack years who either have normal lung function or COPD.

  We hypothesize that young susceptible individuals and COPD patients demonstrate a higher degree and different type of local and systemic inflammation at baseline.
- To study systemic and local inflammation after acute smoke exposure in the above groups. We hypothesize that young susceptible individuals and COPD patients demonstrate a higher and aberrant local and systemic inflammatory response to cigarette smoke.
- To compare in bronchial epithelial cells and PBMCs corticosteroid responsiveness *in vitro* between susceptible and non-susceptible individuals. To study in these cells the effects of cigarette smoking and to elucidate underlying mechanisms of corticosteroid unresponsiveness.
- To determine whether the type and severity of the systemic inflammatory response contributes to the clinical outcome of COPD. We hypothesize that the type and severity of systemic inflammation have profound effects on the clinical picture of COPD.
- To investigate the relationship between downstream genetic effects (transcriptome, proteome) and specific COPD phenotypes in peripheral blood and lung tissue (induced sputum, bronchial biopsies, epithelial lining fluid).

METHODS

Study population

In total 200 old and young individuals who are susceptible or not susceptible to develop COPD will be recruited (table 1). At old age (>40 years), 30 healthy smokers (>20 pack years) and 110 COPD patients (>10 pack years) will be enrolled in the study. At young age, 60 “party smokers” with a normal lung function will be included with a high or low prevalence of COPD in smoking family members (see table 1). Party smoking was defined by irregularly smoking and/or able to quit smoking for at least two days. Exclusion criteria are: α-1-anti-trypsin-deficiency, acute pulmonary infections (like tuberculosis, pneumonia, flue, tracheo-bronchitis), prior history of significant inflammatory lung disease other than COPD (sarcoidosis, pulmonary fibrosis, silicosis, ect.), active infections (such as hepatitis A-C, cystitis, gastro-enteritis etc.), treatment with antibiotics or corticosteroids within 8 weeks, taking part in another study, recent diagnosis of cancer. Medication such as NSAIDs and immunosuppressive agents which could affect the results of the study will be excluded, as well as substance abuse. Co-morbidities that might lead to study-related (serious) adverse events will be excluded on basis of an arbitrary selection of conditions listed in the ACE-27 co-morbidity scale(9).

Table 1 Study population

Disease	No	Age (Yrs)	Smoking status	Pack years	FEV_1 /VC,%	FEV_1, % pred
Non-susceptible						
Healthy [A]	30	18-40	Party smoking	0-10	> 70	> 85
Healthy [B]	30	40-75	Ex or current	>20	> 70	> 85
Susceptible						
Healthy [C]	30	18-40	Party smoking	0-10	> 70	> 85
COPD						
Stage I [D1]	30	40-75	Ex or current	>10	≤ 70	> 80
Stage II [D2]	30	40-75		>10	≤ 70	50-80
Stage III [D3]	30	40-75		>10	≤ 70	30-50
Stage IV [D4]*	20	40-75		>10	≤ 70	< 30
		< 53				< 30%

*Susceptibility in young individuals is based on family history. Not susceptible means that none of the smoking family members who are at least 40 years of age have COPD. Susceptible means that the prevalence of COPD in smoking family members older than 40 years is high: 2 out of 2, 2 out of 3 or 3 out of 3, 3 out of 4 or 4 out of 4. Party smoking was defined by irregularly smoking and/or able to quit smoking for at least two days. Alpha-1-antitrypsin deficiency is excluded. *patients with a FEV_1 30-50% predicted in combination with chronic respiratory failure also have stage IV.*

Study design

This study is a bi-center cross-sectional study that takes place at the University Medical Centers Utrecht (UMC Utrecht) and Groningen (UMC Groningen). Participating subjects will undergo extensive clinical characterisation (table 2). Local and systemic inflammation will be investigated in several ways. Special attention will be paid to acute smoking and corticosteroid insensitivity in selected subgroups.

Table 2 Measurements

	Measurements	Group
Clinical	Demographics	All
	Physical examination	All
	Peripheral blood (routine measurements)	All
	Presence of metabolic syndrome	All
	ECG	B, D1-4
	Bode index	B, D1-4
	Fagerstrom Smoking Questionnaire	All
	St Georges Respiratory Questionnaire (SGRQ)	D1-4
	Clinical COPD Questionnaire (CCQ)	D1-4
	SQUASH	All
	Urine (microproteins)	All
	AGE (Advanced Glycation Endproducts)-reader	All
	Skin blanching test	All
Physiological	Flow volume + reversibility	All
	Body plethysmography	All
	CO diffusion	All
	Methacholine challenge test	A,B,C,D1-3
	Bioelectrical impedance	All
	Six minute walking distance	B,D1-4
Immunological	Sputum induction (only baseline)	A,B,C,D2
	Peripheral blood (systemic inflammation)	All
	Peripheral blood 4x (acute smoking)	A,B,C,D2
	Exhaled breath condensate 3x (acute smoking)	A,B,C,D2
	Exhaled CO 5x (acute smoking)	A,B,C,D2
	Bronchial biopsy 2x (acute smoking)	A,B,C,D2
	Epithelial lining fluid 2x (acute smoking)	A,B,C,D2
	Epithelial brushes 2x (acute smoking)	A,B,C,D2
Radiographical	Low dose HRCT-scan lung	All

Clinical outcomes

Demographic variables include: age, sex, smoking habits, education, profession, other exposures, height and weight. Risk factors of the metabolic syndrome will be determined including blood pressure, waist hip circumference, lipid profile and fasting glucose (table 2). Questionnaires will be the Clinical COPD Questionnaire (CCQ), the St Georges Respiratory Questionnaire (SGRQ), the Dutch Fagerstrom test for nicotine dependence, and the Short QUestionnaire to ASsess Health-enhancing physical activity (SQUASH)(10-12). Exacerbation frequency will be recorded in COPD patients. The BODE-index will be calculated on basis of FEV_1, six-minute-walking distance, Body Mass Index (BMI) and MRC-dyspnea score(4). In urine (micro) protein concentration will be assessed. Corticosteroid sensitivity will be measured by the cutaneous vasoconstrictor response to topical budesonide using the skin blanching test(13). Budesonide dissolved in 95% ethanol will be applied to the skin using eight different concentrations (0-1000 µg/ml). Blanching will be scored with a 7-point scale: 0-3 (increasing with steps of 0.5; 0 = no blanching and 3 = intense blanching). Cumulative oxidative stress will be measured in the skin using the non-invasive AGE (Advanced Glycation Endproducts) reader (DiagnOptics, Groningen, The Netherlands)(14).

Physiological outcomes

Spirometry will be performed according to international guidelines (ERS 2005)(15). We will assess FEV_1, FEV_1/FVC, IVC, FEF50, FEF75, reversibility to salbutamol, TLC, FRC (body box), and CO diffusion. Methacholine challenge tests are performed according to international guidelines (ERS 2005), using serial doubling concentrations of methacholine-bromide (0.03 to 38.4 mg/ml) with the 2-min tidal breathing method at 5-minute intervals. The six-minute-walking-distance (6MWD) will be determined according the American Thoracic Society published guidelines of 2002(16). Individuals should walk at their own pace, can stop if necessary, and are allowed to use oxygen. Body composition will be estimated using single frequency (50 kHz) bioelectrical impedance (Biostat 500), and fat-free-mass will be calculated with the disease-specific equation of Schols et al(17).

Immunological outcomes

Lung inflammation

- Sputum will be induced and processed according to a validated and standardized technique(18), with some modifications. Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells will be performed on May Grünwald Giemsa (MGG) stained cytospins by a qualified cytopathologist.
- Exhaled breath condensate (EBC) will be collected using The EcoScreen® (Jaeger, Hoechberg, Germany). Hydrogen peroxide, pH, 8-isoprostane, nitrite, nitrate, 4-hydroxy-2-nonenal and malondialdehyde will be measured.
- Bronchoscopy will be performed using established guidelines(19-21), and 6 bronchial biopsies will be taken from subsegmental carinae in the right or left lower lobe. Epithelial morphology, epithelial proliferation, and basement membrane thickness will be measured(22). Submucosal density of inflammatory cells (AA1, EG2, CD68, CD3, CD4, CD4CD25, CD8, mast cells, neutrophils) will be quantitated in a semi-automated way(22). Expression of E-Cadherin, VEGF, ICAM, VCAM, E-selection, P-selectin, AGEs and RAGEs will be measured.
- Epithelial lining fluid will be sampled by advancing 3 microsample probes (BC -401C, Olympus, Tokyo, Japan) in the lumen of the left main bronchus (23,24). Cytokines will be measured by Luminex (Linco, Nuclilab BV, Ede, The Netherlands). 90% of the ELF will be used for proteomic analysis. Briefly, each trypsin digested sample will be labeled (iTRAQ® Reagent 8-plex, ABSciex, Foster City, CA, USA) according to the manufacturer's protocol. The individually labeled digests will be combined into a single sample mixture and subjected to strong-cation exchange chromatography (AKTA Purifier, GE Healthcare Biosciences AB, Uppsala, Sweden). The resulting peptide-containing fractions will be separated by reversed-phase chromatography (Ultimate 3000 nanoflow liquid chromatography system, Dionex, Amsterdam, The Netherlands). Fractions of 12 sec will be spotted on MALDI targets (Probot, Dionex, Amsterdam, The Netherlands) and mass spectrometric analysis will be carried out on a 4800 Proteomics Analyzer MALDI

TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA) controlled by the 4000 Series Explorer v3.5 software. Proteins will be identified using Protein Pilot® software v2.0 (Applied Biosystems).

- Bronchial epithelial cells will be harvested from the right or left main bronchus by brushing as described elsewhere(25). Brushed epithelial cells will be cultured to enable corticosteroid sensitivity experiments. In these experiments, cultured bronchial epithelial cells will be incubated *in vitro* with steroids and the effects on chemokine production (IL-8, GRO-a, RANTES) and MMP/TIMP expression (mRNA) will be established. In addition, in peripheral blood mononuclear cells (PBMC) the following parameters will be studied: 1) plasma levels of chemokines/inflammatory cytokines 2) *In vitro* effects of steroids on TNF-_, IL-1_, IL-10, TGF-_, signaling pathways (western/EMSA), TLRs and CD14 expression as well as genes with a GRE in their promoter, e.g. _2-adrenergic receptor, MAPKP-1, FoxP3 (ELISA/RTPCR).

Systemic inflammation

Systemic inflammation will be measured in peripheral blood using several methods to study systemic activation of innate immune cells at three four different levels:

- Expression of established and newly markers on innate immune cells associated with pre-activation(26,27). The established markers include proteins that are up-regulated on the cell surface upon activation of neutrophils in vitro, and can be measured by flowcytometry: CD11b (Mac-1), CD18 (integrin β2 chain), CD66b (CAECAM-8), CD63 (LAMP-3). New markers directed against active integrins and Fc-receptors have been shown useful in detecting more subtle activation such as induced by cytokines: active Mac-1 (CD11b/clone CBRM1/5 (28)), active β1-integrin chain (CD29/ clone N29 (29)), and active FcγRII (CD32/clones A17 (30)). These latter markers will be used to detect subtle priming signals affecting the function of leukocytes in the peripheral blood.

- Determination of the sensitivity of innate immune cells for stimuli. One of the first changes which can be observed in response to inflammatory stimuli in vivo is a change in sensitivity for innate immune stimuli such as fMLF. Little activation is associated with an enhanced responsiveness, whereas pronounced systemic activation is associated with decreased responsiveness for fMLF (31)). Therefore, the responsiveness of leukocytes for fMLF will be measured as read-out for systemic inflammatory signals in vivo.
- Genomic and proteomic analysis of innate immune cells *in vivo* (32). Total mRNA and proteins are collected from leukocytes and will be analysed by unsupervised genomic and proteomics techniques. Proteomics will be carried out by 2D-DIGE (33).
- Multiplex analysis of the presence of pro-and anti-inflammatory cytokines in plasma/serum. Serum samples will be analysed for the presence of multiple cytokines and chemokines by luminex technology (34).

Systemic inflammation will also be measured in peripheral blood using peripheral blood mononuclear cells (PBMC's):

- Expression of intracellular and cell-surface markers of adaptive immune cells (Th1-cells, Th2-cells, Th17-cells, T_{reg}-cells, B-cells, NK-cells) will be measured by flow cytometry.

Lung and systemic inflammation after acute smoking

Young and old subjects who are susceptible or not susceptible to develop COPD will smoke 3 cigarettes in 1 hour. Exhaled CO, Bblood samples, and urine, exhaled breath condensate, bronchial biopsies, epithelial lining fluid and epithelial brushes will be collected at baseline and after smoking according the scheme in table 3. Exhaled CO will be measured at baseline to check if individuals did not smoke recently, and after smoking to check if individuals inhaled cigarette smoke sufficiently. A first bronchoscopy will be performed after 24 hours. Bronchial biopsies, epithelial brushes and microprobe sampling of epithelial lining fluid will be collected. Six weeks after the acute smoking procedure a second bronchoscopy will be performed as a baseline measurement, obtaining the same specimen.

Sputum supernatant, epithelial cells, serum, plasma, DNA, and RNA of blood will be stored for further analyses.

Table 3 Acute smoke model

	Baseline	Smoking 3 cigarettes	5 minutes	2 hours	24 hours	6 weeks
Exhaled CO	x		x	x	x	x
Blood	x			x		
Exhaled breath condensate	x		x	x		
Urine	x			x	x	
Bronchial Biopsies					x	x
Epithelial brush					x	x
Microsampling probe (ELF)					x	x

Samples collected at baseline and during the acute smoking procedure including sputum supernatant, serum, plasma, DNA and RNA of blood, urine, exhaled breath condensate, epithelial lining fluid, epithelial brushes, and bronchial biopsies will be stored for further analyses.

Radiological outcomes

All subjects will undergo a low-dose CT-scan at full inspiration and expiration. Exposure settings will be 30 mAs at 90 kVp for patients weighing less then 50 kg, 30 mAs at 120 kVp for patients weighing between 50 and 80 kg and 30 mAs at 140 kVp for those weighing more than 80 kg without dose modulation. During expiration the exposure settings will be 20 mAs at 90 kVp (body mass < 80kg) or 20 mAs at 120 kVp (body mass > 80kg). Emphysematous lung changes will be quantitated using automated software on low-dose CT scanning images developed in the UMC Utrecht.

Sample size calculation

We concluded that the limited data in the literature do not allow to calculate a reliable sample size according to a formal power-analysis. In general 20-30 subjects per group are needed in studies to detect a significant pro- or anti-inflammatory effect in sputum, BAL or bronchial biopsies. Looking to the available acute smoking studies in the literature this seems sufficient to detect an effect at least in exhaled breath condensate.

Statistical analyses

Demographic variables as age, sex, smoking habits, education, work, other exposures, height and weight will be expressed as means (SD) or medians (IQR) as appropriate for continuous variables, and number (percentages) for dichotomous variables, according to group. Exacerbation frequency will be described (with percentage) per groups. Spirometry data (FEV_1, FEV_1/FVC, IVC, FEF_{50}, FEF_{75}, reversibility to salbutamol, TLCO TLC, FRC (body box), CO diffusion, methacholine challenge tests), and data indicative of systemic inflammation will be described likewise.

Comparisons between groups with regard to all of the above mentioned variables will be tested using Chi-square tests in case of comparison of proportions, and parametric (like the unpaired t-test) or non-parametric tests (like the M-W-U-test/ Wilcoxon rank sum) as appropriate according to the distribution of the residuals. To test changes within groups over time at various visits, additionally paired variants of the before mentioned tests will be used as appropriate (for example, the paired-t-test and the Wilcoxon signed rank test).

Linear or logistic regression will be used to further analyze differences between groups in the above mentioned outcome variables taking confounding factors into account. Techniques like Linear Mixed Effects models will be used to estimate changes in variables over time.

Ethics and dissemination

The two studies are registered at clinicaltrial.gov (identifier study 1: NCT00807469 and identifier study 2: NCT 00850863). These two studies have been judged by the medical ethical committee of UMC Groningen and additionally study 2 has been minimally judged by the medical ethical committee of UMC Utrecht. The study findings will be presented at conferences and will be reported in peer-reviewed journals.

DISCUSSION

There is a large backlog in the recognition of different phenotypes of COPD and their underlying immunopathological processes. This importantly hinders the appropriate diagnosis, treatment and prognosis of this disabilitating disease. Currently lung function (FEV_1 and FEV_1/FVC) is still the standard for the diagnosis and classification of COPD(1). However, there is general consensus that FEV_1 poorly correlates with important patient-centred outcomes such as quality of life, symptoms and exercise capacity(35). Celli et al showed an association between FEV_1 and mortality when FEV_1 was combined with MRC-dyspnoea score, 6-minute walking distance and BMI. The so-called BODE index was put forward as a composite measure to characterise COPD in a more realistic way(4). In the last decades different approaches have been put forward to characterize COPD leading to at least 16 different phenotypes(36). Although clinically relevant in terms of presentation, triggers and treatment response these phenotypes do not necessarily give insight into the underlying disease processes of COPD. In this perspective the term intermediate phenotype or endotype has been put forward to describe a subtype of a disease which is defined by a distinct functional or pathophysiological mechanism(37). Together with genetic and environmental factors intermediate phenotypes may explain the clinical presentation of a heterogeneous disease like COPD. Accordingly, the present study will phenotype the induction and progression of COPD and associate this with underlying pathophysiological mechanisms in a biased as well as non-biased way. As smoking is the most important environmental risk factor for COPD we will use an acute smoking model to evaluate differences in smoking-induced acute mechanisms differentially expressed between individuals with a high and low risk for development of COPD.

Recently, a large prospective cohort study (ECLIPSE) was initiated to study the natural course of COPD in order to gain more insight in the underlying pathogenetic mechanisms(38). The ECLIPSE study is a three year observational study including current and ex-smoking COPD patients and healthy controls with and without a smoking history.

Indeed the ECLIPSE study confirmed that the clinical manifestations of COPD are highly variable and that the degree of airflow limitation does not capture the heterogeneity of the disease(39). Particularly, the rate of change in FEV_1 among patients with COPD was highly variable, with increased rates of decline among current smokers, patients with bronchodilator reversibility and with emphysema(40). Several new susceptibility genes have been identified in the ECLIPSE study(41,42), as well as potentially useful biomarkers(43-45). However, in contrast to our study ECLIPSE does not include young subjects and is, therefore, not able to investigate the susceptibility for COPD at young age. ECLIPSE investigates aspects of systemic inflammation (CRP, TNF-α, IL-6, IL-8, SDP), but does not investigate the activation state of circulating neutrophils and lymphocytes, nor does it perform unbiased proteomic analyses of epithelial lining fluid and peripheral blood neutrophils. Therefore, our study will complement ECLIPSE data by focusing on the pathogenesis of local and systemic inflammation by using unique approaches to link genomic and inflammatory phenotypes in all stages of COPD from preclinical to advanced disease.

The present study has already been started and recruitment is still ongoing. The study population has been described in ClinicalTrial.gov (NCT00807469, NCT 00850863) and was divided into 9 groups. Initially, we planned to distinguish susceptible individuals into a “susceptible” and “very susceptible” group. The group of “old” very susceptible individuals should include early-onset COPD (FEV_1/FVC<70%, FEV_1< 40% predicted, age<53 years) and COPD with low number of pack years (FEV_1/FVC < 70%, FEV_1 < 80%predicted, pack years<5). The group of “young” very susceptible individuals should have included young individuals with family members with early-onset COPD or COPD with low smoke exposure. Despite an intensive search among lung transplantation (LTx) candidates/recipients and their family members we were not able to recruit this group in sufficiently high numbers. Therefore, we decided to combine the susceptible and very susceptible groups.

COPD is often accompanied by different co-morbidities, especially cardiovascular conditions, which also affect the prognosis of the disease as well as quality of life and cost of COPD(46,47). Consequently, we do not exclude subjects with cardiovascular co-morbidity conditions unless the condition was acute or too severe. We use the selected grade 1-3 co-morbidity list in the ACE-27(9) to exclude patients with co-morbidities within grade 2 or 3 in all organ systems except the respiratory system. We also exclude subjects with systemic inflammatory diseases such as rheumatoid arthritis, because we might investigate systemic inflammation related to other systemic inflammatory diseases.

In conclusion this study will provide valuable information regarding the pathogenetic mechanisms underlying the development of COPD, which in the future will help us to develop new targets for the management of different phenotypes of COPD.

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Competing Interests

AT, SH, LF, RB, RH, IH, AO, MB, JWL, and LK have no competing interests to declare. WT has received payment for lectures from Chiesi, GSK, and Roche diagnostics. DP has received an unrestricted educational grant for research from AstraZeneca and Chiesi; fees for consultancies by DP were given to the University of Groningen by AZ, Boehringer Ingelheim, Chiesi, GSK, Nycomed and TEVA; NH received grants from GlaxoSmithKline, Boehringer Ingelheim, Nycomed and Chiesi.

Authors' contributions

NH, JWL, DSP, LK, RB, AO, WT, and MB participated in the design and supervision of the study. ALT and SH were involved in the patient-related investigations and contributed equally to the manuscript. LF performed the proteomic analyses of epithelial lining fluid. RH investigated in vitro corticosteroid sensitivity of epithelial cells. All authors read and approved the final manuscript.

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Figure 1 Acute smoking effects in the lung

Figure 2 General hypothesis about the role of systemic inflammation

Acute and chronic inflammatory responses induced by smoking in individuals being susceptible and non-susceptible for development of COPD: from specific disease phenotyping towards novel therapy: protocol of a cross-sectional study

Short title: Acute and chronic smoking effects and susceptibility to COPD

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ABSTRACT (*Word count = 300)*

Introduction: Chronic obstructive pulmonary disease (COPD) is a heterogeneous disease with pulmonary and extra-pulmonary manifestations. Although COPD is a complex disease, the diagnosis and staging are still based on simple spirometry measurements. Different COPD phenotypes exist based on clinical, physiological, immunological, and radiological observations. Cigarette smoking is the most important risk factor for COPD, but only 15-20% of smokers develop the disease, suggesting a genetic predisposition. Unfortunately, little is known about the pathogenesis of COPD, and even less on the very first steps that are associated with an aberrant response to smoke exposure.

This study aims to investigate the underlying local and systemic inflammation of different clinical COPD phenotypes, and acute effects of cigarette smoke exposure in individuals susceptible and non-susceptible for the development of COPD. Furthermore, we will investigate mechanisms associated with corticosteroid insensitivity. Our study will provide valuable information regarding the pathogenetic mechanisms underlying the natural course of COPD.

Methods and analysis: This cross-sectional study will include young and old individuals susceptible or non-susceptible to develop COPD. At young age (18-40 years) 60 “party smokers” will be included that are called susceptible or non-susceptible based on COPD prevalence in smoking family members. Additionally, 30 healthy smokers (age 40-75 years) and 110 COPD patients will be included. Measurements will include questionnaires, pulmonary function, low-dose CT scanning of the lung, body composition, 6-min-walking distance, and biomarkers in peripheral blood, sputum, urine, exhaled breath condensate, epithelial lining fluid, bronchial brushes and biopsies. Non-biased approaches such as proteomics will be performed in blood and epithelial lining fluid.

Ethics and dissemination: This multicenter study was approved by the medical ethical committees of UMC Groningen and Utrecht, the Netherlands. The study findings will be presented at conferences and will be reported in peer-reviewed journals.

Trial registration: ClinicalTrials.gov, NCT00807469 (study 1) and NCT00850863 (study 2).

ARTICLE SUMMARY

Article focus

This article describes the study protocol of a cross-sectional study investigating the underlying local and systemic inflammation of different clinical COPD phenotypes, and acute effects of cigarette smoke exposure in individuals susceptible and non-susceptible for the development of COPD.

Key messages

- Young (18-40) and older (40-75) individuals who are susceptible and non-susceptible to develop COPD are included.
- All groups are extensively phenotyped by clinical, physiological, immunological, and radiographical characterisation. Furthermore, effects of acute smoking are studied.

Strengths and limitations of the study

- Extensive characterisation of a well defined study population, providing valuable information regarding the pathogenic mechanisms underlying the natural cause of COPD.
- Recruitment of a population with high and low familiar risk to develop COPD.

BACKGROUND

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality worldwide(1). The disease is characterized by persistent and progressive expiratory airflow obstruction (post-bronchodilator $FEV_1/FVC<0.70$) and its severity is based on FEV_1 %predicted(1). Cigarette smoking is the most important risk factor for COPD in the western world, but only 15-20 % of young smokers will eventually develop the disease, suggesting a genetic predisposition. So far, the genetic background of these susceptible smokers has not been elucidated(2). Unravelling the underlying pathogenetic mechanisms of COPD is difficult because it takes 20-30 years of smoking before susceptible smokers develop established COPD. Also, there is the problem that COPD has many clinical expressions, and we just have started to learn how to phenotype this heterogeneous disease. Finally, there are many other risk factors that may modulate the complex interaction between the genetic background and smoking, like *in utero* events, microbial infections, dietary factors, physical inactivity and pharmacological treatment.

It is well accepted that the spirometry measurements (FEV_1 and FVC) are largely insufficient to diagnose and classify COPD(1). With the increased recognition of the various clinical expressions of COPD, consensus is growing that COPD represents a spectrum of overlapping diseases with important extra-pulmonary consequences. Phenotypes of COPD may be classified according to four domains: clinical, physiological, immunological and radiographical(3).

- Clinical distinctions are generally based on dyspnea scores, frequency of exacerbations, body mass, muscle wasting, corticosteroid responsiveness, depression / anxiety, co-morbidity, and healthy status(4).
- Physiological distinctions may be based on the degree of airflow limitation, decline in lung function, bronchodilator responsiveness, airway hyperresponsiveness, CO diffusion capacity, hyperinflation, body-plethysmography, bio-impedance, and exercise tolerance.

- Immunological features comprise the type and severity of local and systemic immunological processes in the lung and systemic compartment. In blood leukocytes cytokines, and mediators may affect the functionality of extra-pulmonary tissues and organs, leading to COPD-associated co-morbid conditions.
- Radiographic distinctions may be based on the presence of various forms and severity of emphysema, thickened large airways, and small airways abnormality on high-resolution computed tomography scans.

Although systemic inflammation and multi-organ pathology have been put forward as important features of COPD, surprisingly little is known about the underlying pathogenesis. Most COPD studies in this field included small numbers of individuals, focused on more severe stages of COPD, characterized subjects clinically on the basis of few arbitrary pulmonary measurements, did not take into account the genetic background and paid limited attention to different aspects of systemic inflammation. In addition, most studies assumed that assessment of cytokines by multiplex assays (e.g. Luminex) is sufficient to accurately describe the systemic inflammatory response. Unfortunately, many caveats are present that preclude a complete insight in this response, e.g.:

- not all cytokines implicated in COPD are known,
- little effort is taken to measure anti-inflammatory cytokines (the balance between pro- and anti-inflammatory signals will probably determine the extent and type of inflammation),
- different pro-inflammatory cytokines can act as heterologous antagonists (inhibit the effects of other cytokines).
- the kinetics of cytokines is very dynamic and no consensus is present regarding an optimal single time point for blood collection.

In the present study we set out to characterize systemic inflammation by an alternative approach. Innate immune cells will be used as integrators of pro- and anti-inflammatory

signals. We hypothesize that subtle changes in the phenotype of granulocytes and monocytes are caused by an “inflammatory imprinting” of these cells.

Cigarette smoking is the main risk factor for developing COPD. Repetitive acute effects of cigarette smoke exposure may accumulate and after many years lead to irreversible lung damage. To understand the changes in the lung due to chronic smoking we believe that it is important to first investigate the exact immunological responses to an acute smoke exposure event, particularly in “naive” lungs that are not yet affected by chronic smoke exposure. The acute (<24 hours) effects of smoking in humans, animals and cell cultures have been extensively reviewed some years ago by van der Vaart and colleagues(5). If we integrate all available data on acute smoking we are able to construct a hypothetical time frame for the acute effects of smoking (figure 1). One of the very first insults on the bronchial system is by oxidants present in cigarette smoke. After local depletion of anti-oxidants, the first oxidative stress products can be measured within 1 hour. These products will disappear within 6 hours. There is a surprisingly fast influx of inflammatory cells; even faster than the synthesis of some pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-8). The exact time period at which the proteinase / antiproteinase balance is affected is unknown; however, protein degradation is measurable within 6 hours after smoking. Unfortunately, until now, only a few studies have investigated the acute effects of cigarette smoking in humans(5). These studies included only small numbers of individuals, characterized subjects mainly on basis of pulmonary measurements, paid no attention to the genetic background and paid limited attention to different aspects of pulmonary inflammation.

Corticosteroids provide little therapeutic benefit in a relatively large group of COPD patients, despite their broad anti-inflammatory effects. Our goal is to identify common markers in peripheral blood monocytes, skin and lung epithelial cells that might contribute to corticosteroid insensitivity. Recently, the GLUCOLD study demonstrated beneficial effects on airway wall inflammation and decline in lung function yet with large inter-individual

differences(6). *In vitro* studies have shown that the ability of dexamethasone to suppress cytokine release (e.g. IL-8) from alveolar macrophages is impaired in COPD patients as compared to healthy smokers(7). Furthermore, alveolar macrophages from healthy smokers are more resistant to corticosteroids than macrophages from non-smokers(8). This relative steroid insensitivity may, in part, be explained by a suppressive effect of cigarette smoke-induced oxidative stress. This suppression may particularly play a role in the airway epithelium, where cells are in first contact with cigarette smoke and form an important source of mediators involved in the induction of neutrophilic airway inflammation (e.g. the chemoattractant IL-8). It may well be that corticosteroid insensitivity is gradually acquired by smoking in COPD, and one might hypothesize that smokers who develop COPD are more prone to have signs of corticosteroid insensitivity.

General hypotheses

There is a clear need to better understand all factors that contribute to the development of COPD and its different phenotypes. This study focuses on the pathogenesis and clinical expression of smoking-induced COPD, studied both in the pulmonary and the systemic compartments. The following general hypothesis is put forward by our consortium (figure 2):

"COPD is a multi-organ disease situated in both the lung and extra-pulmonary organs and tissues. Dysfunction of the latter tissues is exemplified by muscle atrophy, impaired muscle oxidative capacity, osteoporosis, atherosclerosis and heart failure. A low-grade systemic inflammation plays a pivotal role in the induction and perpetuation of this multi-organ disease. Smoking and persistent production of inflammatory mediators from the lung are inducers of systemic inflammation. Other risk factors such as diet deficiencies, sedentary life style, and frequent infections contribute independently to further amplification of systemic inflammation. In more advanced COPD the extra-pulmonary pathology starts to contribute to disease severity and a vicious circle of persistent difficulty to treat inflammation. Consequently, local and systemic inflammation should be reduced in all stages of disease by

reversing negative life style factors and applying successful anti-inflammatory treatment modalities. In more advanced stages multimodal interventions additionally should improve impaired tissue functions. An important contributing problem is the relative corticosteroid insensitivity of both lung and peripheral tissue responses in COPD."

Aims of the study

- To assess systemic and local inflammation at baseline in:
    a) young healthy individuals with low number of pack years smoking who have a high and low familial risk to develop COPD;
    b) older individuals with higher number of pack years who either have normal lung function or COPD.

  We hypothesize that young susceptible individuals and COPD patients demonstrate a higher degree and different type of local and systemic inflammation at baseline.
- To study systemic and local inflammation after acute smoke exposure in the above groups. We hypothesize that young susceptible individuals and COPD patients demonstrate a higher and aberrant local and systemic inflammatory response to cigarette smoke.
- To compare in bronchial epithelial cells and PBMCs corticosteroid responsiveness *in vitro* between susceptible and non-susceptible individuals. To study in these cells the effects of cigarette smoking and to elucidate underlying mechanisms of corticosteroid unresponsiveness.
- To determine whether the type and severity of the systemic inflammatory response contributes to the clinical outcome of COPD. We hypothesize that the type and severity of systemic inflammation have profound effects on the clinical picture of COPD.
- To investigate the relationship between downstream genetic effects (transcriptome, proteome) and specific COPD phenotypes in peripheral blood and lung tissue (induced sputum, bronchial biopsies, epithelial lining fluid).

METHODS

Study population

In total 200 old and young individuals who are susceptible or not susceptible to develop COPD will be recruited (table 1). At old age (>40 years), 30 healthy smokers (>20 pack years) and 110 COPD patients (>10 pack years) will be enrolled in the study. At young age, 60 “party smokers” with a normal lung function will be included with a high or low prevalence of COPD in smoking family members (see table 1). Party smoking was defined by irregularly smoking and/or able to quit smoking for at least two days. Exclusion criteria are: α-1-anti-trypsin-deficiency, acute pulmonary infections (like tuberculosis, pneumonia, flue, tracheo-bronchitis), prior history of significant inflammatory lung disease other than COPD (sarcoidosis, pulmonary fibrosis, silicosis, ect.), active infections (such as hepatitis A-C, cystitis, gastro-enteritis etc.), treatment with antibiotics or corticosteroids within 8 weeks, taking part in another study, recent diagnosis of cancer. Medication such as NSAIDs and immunosuppressive agents which could affect the results of the study will be excluded, as well as substance abuse. Co-morbidities that might lead to study-related (serious) adverse events will be excluded on basis of an arbitrary selection of conditions listed in the ACE-27 co-morbidity scale(9).

Table 1 Study population

Disease	No	Age (Yrs)	Smoking status	Pack years	FEV_1 /VC,%	FEV_1, % pred
Non-susceptible						
Healthy [A]	30	18-40	Party smoking	0-10	> 70	> 85
Healthy [B]	30	40-75	Ex or current	>20	> 70	> 85
Susceptible						
Healthy [C]	30	18-40	Party smoking	0-10	> 70	> 85
COPD						
Stage I [D1]	30	40-75	Ex or current	>10	≤ 70	> 80
Stage II [D2]	30	40-75		>10	≤ 70	50-80
Stage III [D3]	30	40-75		>10	≤ 70	30-50
Stage IV [D4]*	20	40-75		>10	≤ 70	< 30
		< 53				< 30%

*Susceptibility in young individuals is based on family history. Not susceptible means that none of the smoking family members who are at least 40 years of age have COPD. Susceptible means that the prevalence of COPD in smoking family members older than 40 years is high: 2 out of 2, 2 out of 3 or 3 out of 3, 3 out of 4 or 4 out of 4. Party smoking was defined by irregularly smoking and/or able to quit smoking for at least two days. Alpha-1-antitrypsin deficiency is excluded. *patients with a FEV_1 30-50% predicted in combination with chronic respiratory failure also have stage IV.*

Study design

This study is a bi-center cross-sectional study that takes place at the University Medical Centers Utrecht (UMC Utrecht) and Groningen (UMC Groningen). Participating subjects will undergo extensive clinical characterisation (table 2). Local and systemic inflammation will be investigated in several ways. Special attention will be paid to acute smoking and corticosteroid insensitivity in selected subgroups.

Table 2 Measurements

	Measurements	Group
Clinical	Demographics	All
	Physical examination	All
	Peripheral blood (routine measurements)	All
	Presence of metabolic syndrome	All
	ECG	B, D1-4
	Bode index	B, D1-4
	Fagerstrom Smoking Questionnaire	All
	St Georges Respiratory Questionnaire (SGRQ)	D1-4
	Clinical COPD Questionnaire (CCQ)	D1-4
	SQUASH	All
	Urine (microproteins)	All
	AGE (Advanced Glycation Endproducts)-reader	All
	Skin blanching test	All
Physiological	Flow volume + reversibility	All
	Body plethysmography	All
	CO diffusion	All
	Methacholine challenge test	A,B,C,D1-3
	Bioelectrical impedance	All
	Six minute walking distance	B,D1-4
Immunological	Sputum induction (only baseline)	A,B,C,D2
	Peripheral blood (systemic inflammation)	All
	Peripheral blood 4x (acute smoking)	A,B,C,D2
	Exhaled breath condensate 3x (acute smoking)	A,B,C,D2
	Exhaled CO 5x (acute smoking)	A,B,C,D2
	Bronchial biopsy 2x (acute smoking)	A,B,C,D2
	Epithelial lining fluid 2x (acute smoking)	A,B,C,D2
	Epithelial brushes 2x (acute smoking)	A,B,C,D2
Radiographical	Low dose HRCT-scan lung	All

Clinical outcomes

Demographic variables include: age, sex, smoking habits, education, profession, other exposures, height and weight. Risk factors of the metabolic syndrome will be determined including blood pressure, waist hip circumference, lipid profile and fasting glucose (table 2). Questionnaires will be the Clinical COPD Questionnaire (CCQ), the St Georges Respiratory Questionnaire (SGRQ), the Dutch Fagerstrom test for nicotine dependence, and the Short QUestionnaire to ASsess Health-enhancing physical activity (SQUASH)(10-12). Exacerbation frequency will be recorded in COPD patients. The BODE-index will be calculated on basis of FEV_1, six-minute-walking distance, Body Mass Index (BMI) and MRC-dyspnea score(4). In urine (micro) protein concentration will be assessed. Corticosteroid sensitivity will be measured by the cutaneous vasoconstrictor response to topical budesonide using the skin blanching test(13). Budesonide dissolved in 95% ethanol will be applied to the skin using eight different concentrations (0-1000 µg/ml). Blanching will be scored with a 7-point scale: 0-3 (increasing with steps of 0.5; 0 = no blanching and 3 = intense blanching). Cumulative oxidative stress will be measured in the skin using the non-invasive AGE (Advanced Glycation Endproducts) reader (DiagnOptics, Groningen, The Netherlands)(14).

Physiological outcomes

Spirometry will be performed according to international guidelines (ERS 2005)(15). We will assess FEV_1, FEV_1/FVC, IVC, FEF50, FEF75, reversibility to salbutamol, TLC, FRC (body box), and CO diffusion. Methacholine challenge tests are performed according to international guidelines (ERS 2005), using serial doubling concentrations of methacholine-bromide (0.03 to 38.4 mg/ml) with the 2-min tidal breathing method at 5-minute intervals. The six-minute-walking-distance (6MWD) will be determined according the American Thoracic Society published guidelines of 2002(16). Individuals should walk at their own pace, can stop if necessary, and are allowed to use oxygen. Body composition will be estimated using single frequency (50 kHz) bioelectrical impedance (Biostat 500), and fat-free-mass will be calculated with the disease-specific equation of Schols et al(17).

Immunological outcomes

Lung inflammation

- Sputum will be induced and processed according to a validated and standardized technique(18), with some modifications. Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells will be performed on May Grünwald Giemsa (MGG) stained cytospins by a qualified cytopathologist.
- Exhaled breath condensate (EBC) will be collected using The EcoScreen® (Jaeger, Hoechberg, Germany). Hydrogen peroxide, pH, 8-isoprostane, nitrite, nitrate, 4-hydroxy-2-nonenal and malondialdehyde will be measured.
- Bronchoscopy will be performed using established guidelines(19-21), and 6 bronchial biopsies will be taken from subsegmental carinae in the right or left lower lobe. Epithelial morphology, epithelial proliferation, and basement membrane thickness will be measured(22). Submucosal density of inflammatory cells (AA1, EG2, CD68, CD3, CD4, CD4CD25, CD8, mast cells, neutrophils) will be quantitated in a semi-automated way(22). Expression of E-Cadherin, VEGF, ICAM, VCAM, E-selection, P-selectin, AGEs and RAGEs will be measured.
- Epithelial lining fluid will be sampled by advancing 3 microsample probes (BC -401C, Olympus, Tokyo, Japan) in the lumen of the left main bronchus (23,24). Cytokines will be measured by Luminex (Linco, Nuclilab BV, Ede, The Netherlands). 90% of the ELF will be used for proteomic analysis. Briefly, each trypsin digested sample will be labeled (iTRAQ® Reagent 8-plex, ABSciex, Foster City, CA, USA) according to the manufacturer's protocol. The individually labeled digests will be combined into a single sample mixture and subjected to strong-cation exchange chromatography (AKTA Purifier, GE Healthcare Biosciences AB, Uppsala, Sweden). The resulting peptide-containing fractions will be separated by reversed-phase chromatography (Ultimate 3000 nanoflow liquid chromatography system, Dionex, Amsterdam, The Netherlands). Fractions of 12 sec will be spotted on MALDI targets (Probot, Dionex, Amsterdam, The Netherlands) and mass spectrometric analysis will be carried out on a 4800 Proteomics Analyzer MALDI

TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA) controlled by the 4000 Series Explorer v3.5 software. Proteins will be identified using Protein Pilot® software v2.0 (Applied Biosystems).

- Bronchial epithelial cells will be harvested from the right or left main bronchus by brushing as described elsewhere(25). Brushed epithelial cells will be cultured to enable corticosteroid sensitivity experiments. In these experiments, cultured bronchial epithelial cells will be incubated *in vitro* with steroids and the effects on chemokine production (IL-8, GRO-a, RANTES) and MMP/TIMP expression (mRNA) will be established. In addition, in peripheral blood mononuclear cells (PBMC) the following parameters will be studied: 1) plasma levels of chemokines/inflammatory cytokines 2) *In vitro* effects of steroids on TNF-_, IL-1_, IL-10, TGF-_, signaling pathways (western/EMSA), TLRs and CD14 expression as well as genes with a GRE in their promoter, e.g. _2-adrenergic receptor, MAPKP-1, FoxP3 (ELISA/RTPCR).

Systemic inflammation

Systemic inflammation will be measured in peripheral blood using several methods to study systemic activation of innate immune cells at ~~three~~ four different levels:

- Expression of established and newly markers on innate immune cells associated with pre-activation(26,27). The established markers include proteins that are up-regulated on the cell surface upon activation of neutrophils in vitro, and can be measured by flowcytometry: CD11b (Mac-1), CD18 (integrin β2 chain), CD66b (CAECAM-8), CD63 (LAMP-3). New markers directed against active integrins and Fc-receptors have been shown useful in detecting more subtle activation such as induced by cytokines: active Mac-1 (CD11b/clone CBRM1/5 (28)), active β1-integrin chain (CD29/ clone N29 (29)), and active FcγRII (CD32/clones A17 (30)). These latter markers will be used to detect subtle priming signals affecting the function of leukocytes in the peripheral blood.

- Determination of the sensitivity of innate immune cells for stimuli. One of the first changes which can be observed in response to inflammatory stimuli in vivo is a change in sensitivity for innate immune stimuli such as fMLF. Little activation is associated with an enhanced responsiveness, whereas pronounced systemic activation is associated with decreased responsiveness for fMLF (31)). Therefore, the responsiveness of leukocytes for fMLF will be measured as read-out for systemic inflammatory signals in vivo.
- Genomic and proteomic analysis of innate immune cells *in vivo* (32). Total mRNA and proteins are collected from leukocytes and will be analysed by unsupervised genomic and proteomics techniques. Proteomics will be carried out by 2D-DIGE (33).
- Multiplex analysis of the presence of pro-and anti-inflammatory cytokines in plasma/serum. Serum samples will be analysed for the presence of multiple cytokines and chemokines by luminex technology (34).

Systemic inflammation will also be measured in peripheral blood using peripheral blood mononuclear cells (PBMC's):

- Expression of intracellular and cell-surface markers of adaptive immune cells (Th1-cells, Th2-cells, Th17-cells, T_{reg}-cells, B-cells, NK-cells) will be measured by flow cytometry.

Lung and systemic inflammation after acute smoking

Young and old subjects who are susceptible or not susceptible to develop COPD will smoke 3 cigarettes in 1 hour. Exhaled CO, ~~B~~blood samples, and urine, ~~exhaled breath condensate, bronchial biopsies, epithelial lining fluid and epithelial brushes~~ will be collected at baseline and after smoking according the scheme in table 3. Exhaled CO will be measured at baseline to check if individuals did not smoke recently, and after smoking to check if individuals inhaled cigarette smoke sufficiently. A first bronchoscopy will be performed after 24 hours. Bronchial biopsies, epithelial brushes and microprobe sampling of epithelial lining fluid will be collected. Six weeks after the acute smoking procedure a second bronchoscopy will be performed as a baseline measurement, obtaining the same specimen.

~~Sputum supernatant, epithelial cells, serum, plasma, DNA, and RNA of blood will be stored for further analyses.~~

Table 3 Acute smoke model

	Baseline	Smoking 3 cigarettes	5 minutes	2 hours	24 hours	6 weeks
Exhaled CO	x		x	x	x	x
Blood	x			x		
Exhaled breath condensate	x		x	x		
Urine	x			x	x	
Bronchial Biopsies					x	x
Epithelial brush					x	x
Microsampling probe (ELF)					x	x

Samples collected at baseline and during the acute smoking procedure including sputum supernatant, serum, plasma, DNA and RNA of blood, urine, exhaled breath condensate, epithelial lining fluid, epithelial brushes, and bronchial biopsies will be stored for further analyses.

Radiological outcomes

All subjects will undergo a low-dose CT-scan at full inspiration and expiration. Exposure settings will be 30 mAs at 90 kVp for patients weighing less then 50 kg, 30 mAs at 120 kVp for patients weighing between 50 and 80 kg and 30 mAs at 140 kVp for those weighing more than 80 kg without dose modulation. During expiration the exposure settings will be 20 mAs at 90 kVp (body mass < 80kg) or 20 mAs at 120 kVp (body mass > 80kg). Emphysematous lung changes will be quantitated using automated software on low-dose CT scanning images developed in the UMC Utrecht.

Sample size calculation

We concluded that the limited data in the literature do not allow to calculate a reliable sample size according to a formal power-analysis. In general 20-30 subjects per group are needed in studies to detect a significant pro- or anti-inflammatory effect in sputum, BAL or bronchial biopsies. Looking to the available acute smoking studies in the literature this seems sufficient to detect an effect at least in exhaled breath condensate.

Statistical analyses

Demographic variables as age, sex, smoking habits, education, work, other exposures, height and weight will be expressed as means (SD) or medians (IQR) as appropriate for continuous variables, and number (percentages) for dichotomous variables, according to group. Exacerbation frequency will be described (with percentage) per groups. Spirometry data (FEV_1, FEV_1/FVC, IVC, FEF_{50}, FEF_{75}, reversibility to salbutamol, TLCO TLC, FRC (body box), CO diffusion, methacholine challenge tests), and data indicative of systemic inflammation will be described likewise.

Comparisons between groups with regard to all of the above mentioned variables will be tested using Chi-square tests in case of comparison of proportions, and parametric (like the unpaired t-test) or non-parametric tests (like the M-W-U-test/ Wilcoxon rank sum) as appropriate according to the distribution of the residuals. To test changes within groups over time at various visits, additionally paired variants of the before mentioned tests will be used as appropriate (for example, the paired-t-test and the Wilcoxon signed rank test).

Linear or logistic regression will be used to further analyze differences between groups in the above mentioned outcome variables taking confounding factors into account. Techniques like Linear Mixed Effects models will be used to estimate changes in variables over time.

Ethics and dissemination

The two studies are registered at clinicaltrial.gov (identifier study 1: NCT00807469 and identifier study 2: NCT 00850863). These two studies have been judged by the medical ethical committee of UMC Groningen and additionally study 2 has been minimally judged by the medical ethical committee of UMC Utrecht. The study findings will be presented at conferences and will be reported in peer-reviewed journals.

DISCUSSION

There is a large backlog in the recognition of different phenotypes of COPD and their underlying immunopathological processes. This importantly hinders the appropriate diagnosis, treatment and prognosis of this disabilitating disease. Currently lung function (FEV_1 and FEV_1/FVC) is still the standard for the diagnosis and classification of COPD(1). However, there is general consensus that FEV_1 poorly correlates with important patient-centred outcomes such as quality of life, symptoms and exercise capacity(35). Celli et al showed an association between FEV_1 and mortality when FEV_1 was combined with MRC-dyspnoea score, 6-minute walking distance and BMI. The so-called BODE index was put forward as a composite measure to characterise COPD in a more realistic way(4). In the last decades different approaches have been put forward to characterize COPD leading to at least 16 different phenotypes(36). Although clinically relevant in terms of presentation, triggers and treatment response these phenotypes do not necessarily give insight into the underlying disease processes of COPD. In this perspective the term intermediate phenotype or endotype has been put forward to describe a subtype of a disease which is defined by a distinct functional or pathophysiological mechanism(37). Together with genetic and environmental factors intermediate phenotypes may explain the clinical presentation of a heterogeneous disease like COPD. Accordingly, the present study will phenotype the induction and progression of COPD and associate this with underlying pathophysiological mechanisms in a biased as well as non-biased way. As smoking is the most important environmental risk factor for COPD we will use an acute smoking model to evaluate differences in smoking-induced acute mechanisms differentially expressed between individuals with a high and low risk for development of COPD.

Recently, a large prospective cohort study (ECLIPSE) was initiated to study the natural course of COPD in order to gain more insight in the underlying pathogenetic mechanisms(38). The ECLIPSE study is a three year observational study including current and ex-smoking COPD patients and healthy controls with and without a smoking history.

Indeed the ECLIPSE study confirmed that the clinical manifestations of COPD are highly variable and that the degree of airflow limitation does not capture the heterogeneity of the disease(39). Particularly, the rate of change in FEV_1 among patients with COPD was highly variable, with increased rates of decline among current smokers, patients with bronchodilator reversibility and with emphysema(40). Several new susceptibility genes have been identified in the ECLIPSE study(41,42), as well as potentially useful biomarkers(43-45). However, in contrast to our study ECLIPSE does not include young subjects and is, therefore, not able to investigate the susceptibility for COPD at young age. ECLIPSE investigates aspects of systemic inflammation (CRP, TNF-α, IL-6, IL-8, SDP), but does not investigate the activation state of circulating neutrophils and lymphocytes, nor does it perform unbiased proteomic analyses of epithelial lining fluid and peripheral blood neutrophils. Therefore, our study will complement ECLIPSE data by focusing on the pathogenesis of local and systemic inflammation by using unique approaches to link genomic and inflammatory phenotypes in all stages of COPD from preclinical to advanced disease.

The present study has already been started and recruitment is still ongoing. The study population has been described in ClinicalTrial.gov (NCT00807469, NCT 00850863) and was divided into 9 groups. Initially, we planned to distinguish susceptible individuals into a “susceptible” and “very susceptible” group. The group of “old” very susceptible individuals should include early-onset COPD (FEV_1/FVC<70%, FEV_1< 40% predicted, age<53 years) and COPD with low number of pack years (FEV_1/FVC < 70%, FEV_1 < 80%predicted, pack years<5). The group of “young” very susceptible individuals should have included young individuals with family members with early-onset COPD or COPD with low smoke exposure. Despite an intensive search among lung transplantation (LTx) candidates/recipients and their family members we were not able to recruit this group in sufficiently high numbers. Therefore, we decided to combine the susceptible and very susceptible groups.

COPD is often accompanied by different co-morbidities, especially cardiovascular conditions, which also affect the prognosis of the disease as well as quality of life and cost of COPD(46,47). Consequently, we do not exclude subjects with cardiovascular co-morbidity conditions unless the condition was acute or too severe. We use the selected grade 1-3 co-morbidity list in the ACE-27(9) to exclude patients with co-morbidities within grade 2 or 3 in all organ systems except the respiratory system. We also exclude subjects with systemic inflammatory diseases such as rheumatoid arthritis, because we might investigate systemic inflammation related to other systemic inflammatory diseases.

In conclusion this study will provide valuable information regarding the pathogenetic mechanisms underlying the development of COPD, which in the future will help us to develop new targets for the management of different phenotypes of COPD.

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Competing Interests

AT, SH, LF, RB, RH, IH, AO, MB, JWL, and LK have no competing interests to declare. WT has received payment for lectures from Chiesi, GSK, and Roche diagnostics. DP has received an unrestricted educational grant for research from AstraZeneca and Chiesi; fees for consultancies by DP were given to the University of Groningen by AZ, Boehringer Ingelheim, Chiesi, GSK, Nycomed and TEVA; NH received grants from GlaxoSmithKline, Boehringer Ingelheim, Nycomed and Chiesi.

Authors' contributions

NH, JWL, DSP, LK, RB, AO, WT, and MB participated in the design and supervision of the study. ALT and SH were involved in the patient-related investigations and contributed equally to the manuscript. LF performed the proteomic analyses of epithelial lining fluid. RH investigated in vitro corticosteroid sensitivity of epithelial cells. All authors read and approved the final manuscript.

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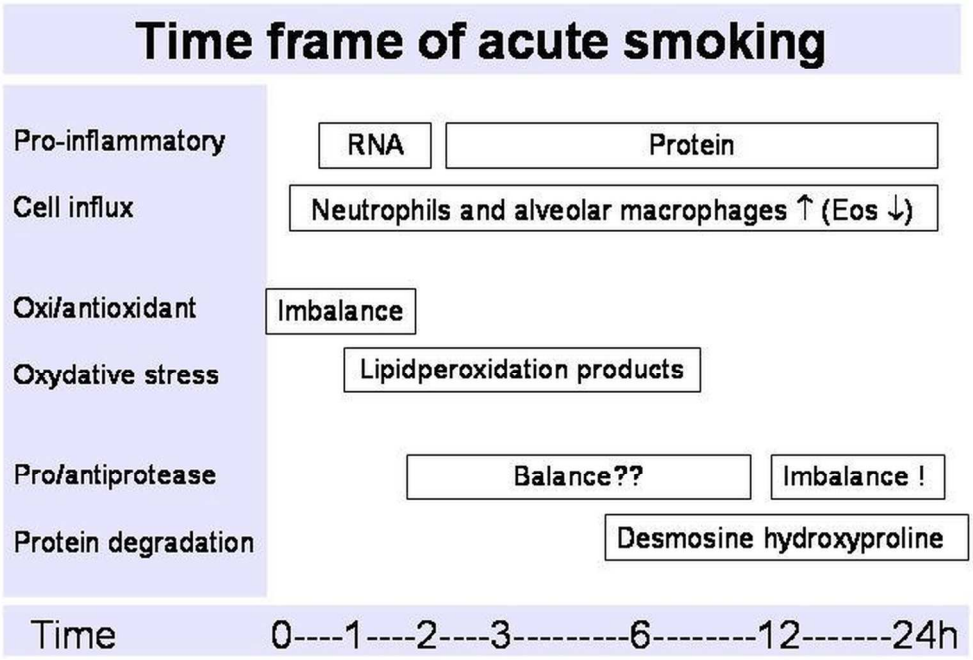
Figure 1 Acute smoking effects in the lung

Figure 2 General hypothesis about the role of systemic inflammation



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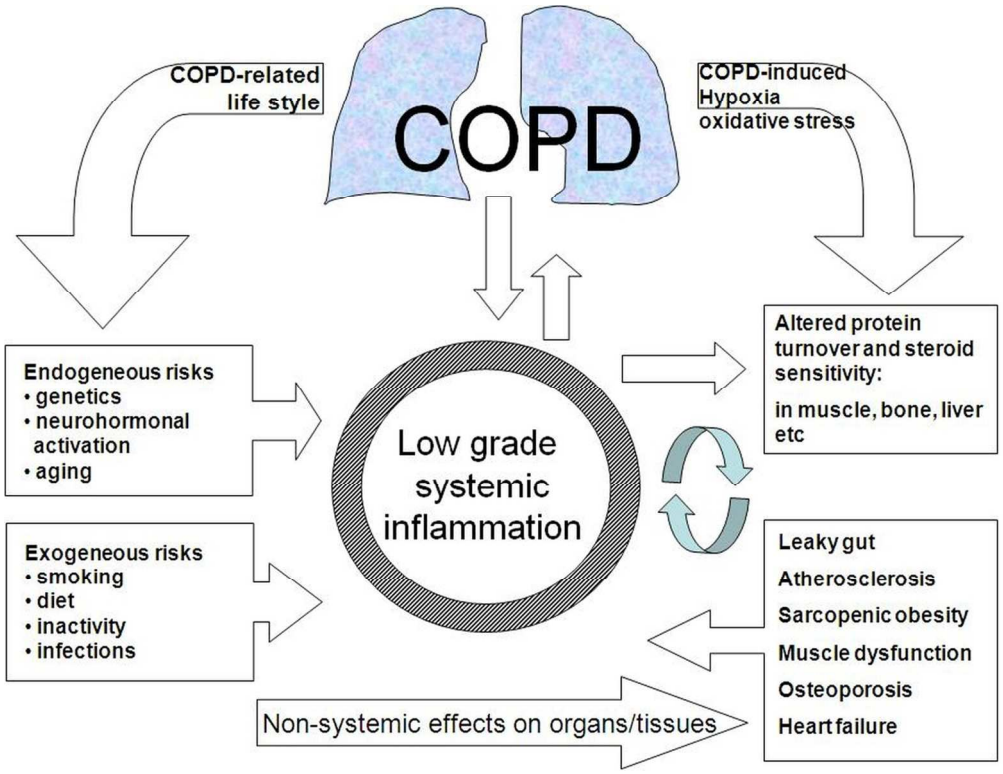

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Groningen, The Netherlands, 17 December 2012

Dear Editor,

Please find attached our revised manuscript entitled **'Acute and chronic inflammatory responses induced by smoking in individuals being susceptible and non-susceptible for development of COPD: from specific disease phenotyping towards novel therapy: a cross-sectional study'.**

We would like to thank the reviewers for their excellent and constructive comments to improve this manuscript. We have modified the text according to the majority of the reviewers' suggestions. Our reply to all the points raised by the reviewers is indicated in the *'Response to decision letter'* document.

We hope that you find this revised manuscript suitable for publication in *BMJ Open*.

Sincerely yours,

On behalf of all authors,

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