PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	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
AUTHORS	Hoonhorst, Susan; Lo Tam Loi, Adèle; Franciosi, Lorenza; Bischoff, Rainer; Hoffmann, Roland; Heijink, Irene; van Oosterhout, Antoon; Boezen, H. Marike; Timens, Wim; Postma, Dirkje; Lammers, Jan-Willem; Koenderman, Leo; ten Hacken, Nick

VERSION 1 - REVIEW

REVIEWER	Prof. Antonio Spanevello Dipartimento di Medicina Clinica e Sperimentale Malattie dell'Apparato Respiratorio Università degli Studi dell'Insubria Dipartimento di Pneumologia Riabilitativa Fondazione Salvatore Maugeri, IRCCS Tradate (VA), Italy
REVIEW RETURNED	05-Nov-2012

THE STUDY	The authors should report how the data will be statistically evaluated and how they calculated the sample size in the study The part regarding the systemic inflammation described in the immunological outcomes is too general, the authors will measure systemic inflammation in peripheral blood using several methods at three different levels; which ones since four points are reported? The authors did not report which established and new markers of pre-activation of innate immune cells they want to study and in which way
GENERAL COMMENTS	The authors presented a really comprehensive protocol in order to evaluate the acute and chronic inflammatory response induced by smoking in COPD susceptible and non-susceptible individuals. Furthermore, they also aimed to study the responsiveness of the enrolled subjects to the corticosteroid therapy. The protocol is really ambitious and it proposes to address the aim with different approaches: clinical, physiological, immunological and radiological. COMMENTS: 1.The protocol would be more complete if the authors include a group of healthy subjects, 40-75 yrs, who have never smoked. Some authors have already described the occurrence of a minimal subclinical airway inflammation associated with aging. This group would help in understanding what is the weight of this part of inflammation on the progression of smoke induced airway inflammation and on the development of COPD.

	2.The authors should also include in the inflammatory evaluations some biomarkers of airway infections and a sputum microbiological culture, considering that the airways of COPD patients could be very frequently colonized or infected and this condition could definitely alter the inflammatory cell and mediator profile. 3.The part regarding the systemic inflammation described in the immunological outcomes is too general, the authors will measure systemic inflammation in peripheral blood using several methods at three different levels; which ones since four points are reported? The authors did not report which established and new markers of pre-activation of innate immune cells they want to study and in which way. 4.In the part related to lung and systemic inflammation after acute smoking, the authors reported sputum supernatant but this methodology isn't present in table 3 as baseline determination. 5.Why will bronchial biopsies, epithelial brush and microsampling probe be performed 24h and 6 weeks after the acute smoking and not at baseline? 6.The authors should better elucidate how they will evaluate the bronchial epithelial cells and PMBC corticosteroid responsiveness. 7.The authors should report how the data will be statistically evaluated and how they calculated the sample size in the study. 8.In the title of the protocol the mention to "novel therapy" is a little bit pretentious, "tailor-made therapy" would be more appropriate.

REVIEWER	LEONARDO M. FABBRI UNIVERSITY OF MODENA AND REGGIO EMILIA
REVIEW RETURNED	20-Nov-2012

THE STUDY	THE PAPER DESCRIBES THE PROTOCOL OF A CROSS SECTIONAL STUDY ON COPD PATIENTS THAT IN MY OPINION IS REDUNDANT CONSIDERING THE MUCH LARGER PUBLISHED (ECLIPSE) AND ONGOING (COPDGENE) PROSPECTIVE STUDIES THAT INCLUDE ALMOST ALL VARIABLES ARE PLANNEND IN THIS STUDY. THE HYPOTHESIS THAT ACUTE RESPONSE TO SMOKING COULD BE PREDICTABLE OF FUTURE DEVELOPMENT OF COPD IN SUSCEPTIBLE INDIVIDUAL IS INTERESTING BUT IT REQUIRES A PROSPECTIVE DESIGN TO BE ADDRESS.

VERSION 1 – AUTHOR RESPONSE

Reviewer: Prof. Antonio Spanevello
Dipartimento di Medicina Clinica e Sperimentale Malattie dell'Apparato Respiratorio Università degli Studi dell'Insubria Dipartimento di Pneumologia Riabilitativa Fondazione Salvatore Maugeri, IRCCS Tradate (VA), Italy

The authors presented a really comprehensive protocol in order to evaluate the acute and chronic inflammatory response induced by smoking in COPD susceptible and non-susceptible individuals. Furthermore, they also aimed to study the responsiveness of the enrolled subjects to the corticosteroid therapy. The protocol is really ambitious and it proposes to address the aim with different approaches: clinical, physiological, immunological and radiological.

COMMENTS:

1. The protocol would be more complete if the authors include a group of healthy subjects, 40-75 yrs, who have never smoked. Some authors have already described the occurrence of a minimal subclinical airway inflammation associated with aging. This group would help in understanding what is the weight of this part of inflammation on the progression of smoke induced airway inflammation and on the development of COPD.

We would like to thank the reviewer for this good suggestion. However, the study has already been started so no changes can be made in the protocol. It is worth mentioning that in the same time period we also started a parallel study (NORM) at our department. In this study healthy smokers and never-smokers at young and old age are extensively characterized collecting i.a. lung function, peripheral blood collection, sputum induction, and bronchial biopsies. All these measurements were performed according to the same protocols, which makes it suitable to use subjects of this study as a control group, as the reviewer suggested.

2. The authors should also include in the inflammatory evaluations some biomarkers of airway infections and a sputum microbiological culture, considering that the airways of COPD patients could be very frequently colonized or infected and this condition could definitely alter the inflammatory cell and mediator profile.

This is a good point. However, the study already has been started, so no changes can be made in the protocol. In the future we might determine micro biome-gene expression in biopsies.

3. The part regarding the systemic inflammation described in the immunological outcomes is too general, the authors will measure systemic inflammation in peripheral blood using several methods at three different levels; which ones since four points are reported? The authors did not report which established and new markers of pre-activation of innate immune cells they want to study and in which way.

We agree with the reviewer that the part regarding the systemic inflammation may be too general. Therefore we expanded each part describing the methods used for analyses, we described the different new markers of pre-activation of innate immune cells we will study, and we added some references. Furthermore, we changed the measurements at three different levels into four different levels (page 14 and 15).

‘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, Treg-cells, B-cells, NK-cells).'

This text was expanded as follow:

'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, Treg-cells, B-cells, NK-cells) will be measured by flow cytometry.'

4. In the part related to lung and systemic inflammation after acute smoking, the authors reported sputum supernatant but this methodology isn't present in table 3 as baseline determination.

We agree with the reviewer, this may be somewhat confusing. Sputum collection is not a part of the acute smoking procedure which is represented in table 3, but will only be performed as a baseline characterization (mentioned in table 2, measurements). We adjusted the protocol at page 16:

The sentence: 'Sputum supernatant, epithelial cells, serum, plasma, DNA, and RNA of blood will be stored for further analyses' was removed and replaced after table 3, and was modified as follow:

Samples collected at baseline and during the acute smoking procedure including sputum supernatant, epithelial cells, 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.

5. Why will bronchial biopsies, epithelial brush and microsampling probe be performed 24h and 6 weeks after the acute smoking and not at baseline?

The bronchoscopy collecting bronchial biopsies, epithelial brush and microsampling probes after 6 weeks is the baseline measurement. We agree that this was not clearly described in the text (page 15):

'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.'

Therefore we adapted the text into the following and hope this makes it more clear:

'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.'

6. The authors should better elucidate how they will evaluate the bronchial epithelial cells and PMBC corticosteroid responsiveness.

We expanded the methods about the corticosteroid sensitivity part in section 'Immunological outcomes', 'lung inflammation' (page 14).

'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.'

This alinea was expanded as follow:

'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).'

7. The authors should report how the data will be statistically evaluated and how they calculated the sample size in the study.

We added two paragraphs in the manuscript at page 17, titled 'Sample size calculation' and 'Statistical analyses':

'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 (FEV1, FEV1/FVC, IVC, FEF50, FEF75, 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 ttest) 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.'

8. In the title of the protocol the mention to "novel therapy" is a little bit pretentious, "tailor-made therapy" would be more appropriate.

We really appreciate the reviewers' suggestion to improve the title of our study protocol. However, the study is registrated and approved at trial registration register and the medical ethic committee with this specific title. Actually, to be consistence and for recognition we prefer to keep the title as it was originally devised.

Reviewer: LEONARDO M. FABBRI
UNIVERSITY OF MODENA AND REGGIO EMILIA

THE PAPER DESCRIBES THE PROTOCOL OF A CROSS SECTIONAL STUDY ON COPD PATIENTS THAT IN MY OPINION IS REDUNDANT CONSIDERING THE MUCH LARGER

PUBLISHED (ECLIPSE) AND ONGOING (COPDGENE) PROSPECTIVE STUDIES THAT INCLUDE ALMOST ALL VARIABLES ARE PLANNEND IN THIS STUDY. THE HYPOTHESIS THAT ACUTE RESPONSE TO SMOKING COULD BE PREDICTABLE OF FUTURE DEVELOPMENT OF COPD IN SUSCEPTIBLE INDIVIDUAL IS INTERESTING BUT IT REQUIRES A PROSPECTIVE DESIGN TO BE ADDRESS.
NO

We would like to thank the reviewer for this comment. However, we do not fully agree.
As mentioned, our study distinguishes from the above described cohorts because of having an acute smoking procedure performed in susceptible and non-susceptible individuals at young and old ages. Although the numbers of our population may be smaller compared with the above described cohorts, we think we have chosen for a unique study design. We did not choose to set-up a long-term prospective design because we were primarily interested in the acute effects (the early effects) of cigarette smoking in our specific research groups. Our assumption was that these early effects differ between individuals who are susceptible and non-susceptible to develop COPD, and therefore might identify the most important aberrant response to smoke responsible for COPD development. Another assumption was that at young age this response to cigarette smoke is not complicated by the structural lung changes present in patients with COPD or healthy subjects with life-long chronic smoking. Once we have identified this very first aberrant response we might study the fate of this response during long-term follow-up, but recognize like dr Fabbri that a prospective study over 2-3 years (like in ECLIPSE) still has important limitations because COPD needs 20-30 years to develop in real life. YES, WE BELIEVE THAT OUR STUDY ADS TO THE LARGE COHORT STUDIES THAT DR FABBRI REFERS TO.

VERSION 2 – REVIEW

REVIEWER	Prof.Antonio Spanevello Dipartimento di Medicina Clinica e Sperimentale Malattie dell'Apparato Respiratorio Università degli Studi dell'Isubria Dipartimento di Pneumologia Riabilitativa Fondazione Salvatore Maugeri, IRCCS Tradate (VA), Italy
REVIEW RETURNED	23-Dec-2012

- The reviewer completed the checklist but made no further comments.